

**PATENT**  
**ATTY DKT: DEKM:177US**

**APPLICATION FOR UNITED STATES LETTERS PATENT**

**for**

**MAIZE CYTOPLASMIC GLUTAMINE SYNTHETASE PROMOTER COMPOSITIONS  
AND METHODS FOR USE THEREOF**

**by**

**BRENDAN HINCHEY**

**and**

**HEE-SOOK SONG**

EXPRESS MAIL NO.: EL 780053335 US  
DATE OF DEPOSIT: November 20, 2001

## **BACKGROUND OF THE INVENTION**

### **1. Field of the Invention**

The present invention relates generally to transgenic plants. More specifically, it relates to methods and compositions for transgene expression using a promoter naturally associated with a *Zea mays* nuclear gene encoding a cytoplasmic glutamine synthase.

### **2. Description of the Related Art**

An important aspect in the production of genetically engineered crops is obtaining sufficient levels of transgene expression in the appropriate plant tissues, especially tissues that are involved in reproductive functions. In this respect, the selection of promoters for directing expression of a given transgene is crucial. Promoters which are useful for plant transgene expression include those that are inducible, viral, synthetic, constitutive as described (Paszkowski *et al.*, 1984; Odell *et al.*, 1985), temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989).

A number of plant promoters have been described with various expression characteristics. Examples of some constitutive promoters which have been described include the rice actin 1 (Wang *et al.*, 1992; U.S. Patent No. 5,641,876), CaMV 35S (Odell *et al.*, 1985), CaMV 19S (Lawton *et al.*, 1987), Ti plasmid nopaline synthase (*nos*, Ebert *et al.*, 1987), alcohol dehydrogenase (*Adh*, Walker *et al.*, 1987), and sucrose synthase (Yang and Russell, 1990).

Examples of tissue specific promoters which have been described include lectin (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989; Dennis *et al.*, 1984), corn light harvesting complex (Simpson, 1986; Bansal *et al.*, 1992), corn heat shock protein (Odell *et al.*, 1985; Rochester *et al.*, 1986), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*,

1988), bean glycine rich protein 1 (Keller *et al.*, 1989), truncated CaMV 35s (Odell *et al.*, 1985), potato patatin (Wenzler *et al.*, 1989), root cell (Conkling *et al.*, 1990), maize zein (Reina *et al.*, 1990; Kriz *et al.*, 1987; Wandelt and Feix, 1989; Langridge and Feix, 1983; Reina *et al.*, 1990), globulin-1 (Belanger and Kriz *et al.*, 1991),  $\alpha$ -tubulin (Carpenter *et al.*, 1992; Uribe *et al.*, 1998), cab (Sullivan *et al.*, 1989), PEPCase (Hudspeth and Grula, 1989), R gene complex-associated promoters (Chandler *et al.*, 1989), chalcone synthase promoters (Franken *et al.*, 1991) and glutamine synthetase promoters (U.S. Patent No. 5,391,725; Edwards *et al.*, 1990; Brears *et al.*, 1991).

Inducible promoters which have been described include ABA- and turgor-inducible promoters, the promoter of the auxin-binding protein gene (Schwob *et al.*, 1993), the UDP glucose flavonoid glycosyl-transferase gene promoter (Ralston *et al.*, 1988); the MPI proteinase inhibitor promoter (Cordero *et al.*, 1994), the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler *et al.*, 1995; Quigley *et al.*, 1989; Martinez *et al.*, 1989) and a light inducible plastid glutamine synthetase gene from pea (U.S. Patent No. 5,391,725; Edwards *et al.*, 1990).

Promoters that are active in functions relating to the development of male or female reproductive tissues as well as seed specific activities have also been described. For example, promoters for anther-specific genes such as *apg* from *Arabidopsis* (Roberts *et al.*, 1993) and *ra8* from rice (Jeon *et al.*, 1999), and pollen-specific genes such as *lat52* and *lat59* from tomato (Twell *et al.*, 1990), and *ZM13* from maize (Hamilton *et al.*, 1992) have been disclosed. Promoters for genes involved in the development of female tissues such as the *Msg* gene from soybean (Stromvik *et al.*, 1999) and the chalcone synthase A gene from petunia (van der Meer *et al.*, 1990) have been reported. Regulatory sequences for genes involved in the development of embryos or endosperm have been disclosed, for example the Brassica napin storage protein promoter (NapA; Ellerstrom *et al.*, 1996) and the Opaque2 promoter from maize (Gallusci *et al.*, 1994). However, while a promoter may be expressed in a reproductive tissue, it may also show regulation in an unrelated tissue; for example the ADH1 promoter of maize was found to express in roots as well as in endosperm tissues (Kyoizuka *et al.*, 1994). Considering the complex regulation that occurs during the formation of reproductive organs in higher plants, relatively few

promoters specifically directing this aspect of development have been identified. It would be of benefit to the art to increase the number and variety of promoters involved in the development of reproductive organs.

5           Glutamine synthetase (EC 6.3.1.2) plays a key role in nitrogen metabolism in a diverse array of organisms including bacteria, humans and plants. More specifically, the enzyme catalyzes the addition of ammonium to glutamate to synthesize glutamine in an ATP-dependent reaction. Bacterial forms of glutamine synthetase (GS) are well characterized, however, the enzyme has received less study in eukaryotes (reviewed in Eisenberg *et al.*, 2000). Clones for several  
10       procaryotic and eukaryotic glutamine synthetase genes have been isolated. Despite significant overall differences at both the nucleotide and protein levels, enzymes from assorted species show highly conserved amino acid residues believed to be important for active site function. This conservation of select residues suggests that the various enzymes function via a similar catalytic mechanism (Eisenberg *et al.*, 2000).

15           In higher plants, glutamine synthetase is found in a variety of tissues, including leaf, root, seed, root nodule and fruit. In addition, there are two forms of glutamine synthetase: a cytosolic form (GS<sub>1</sub>) typically found in roots and leaves, and a plastidic form, primarily found in leaves (GS<sub>2</sub>). The cytosolic form is further characterized as being present in several different isoforms, or isozymes, within a plant.  
20

25           The various isoforms of glutamine synthetase function as members of a complex cycle in the plant, with roles including the detection of inorganic nitrogen sources, ammonium assimilation, incorporation of acquired nitrogen into organic forms and the reassimilation of nitrogen released during metabolism (reviewed in Coruzzi, 1991; McGrath and Coruzzi, 1991; Lam *et al.*, 1996; Stitt, 1999; Oliveira *et al.*, 2001). Thus, glutamine synthetase affects growth, development and overall plant metabolism, and especially carbon metabolism (see McGrath and Coruzzi, 1991; Lam *et al.*, 1996; Stitt, 1999; Oliveira *et al.*, 2001).



Several glutamine synthetase genes have been isolated and all have been found to be encoded by nuclear genes. The plastidic form of the enzyme appears to be coded for by a single gene while the various isoforms of the cytosolic enzymes are coded for by small, multigene families (Tingey *et al.*, 1987; Sakamoto *et al.*, 1989; Brears *et al.*, 1991; Li *et al.*, 1993; Dubois *et al.*, 1996; Lam *et al.*, 1996). The members of the multigene families are believed to encode different subunits which may combine to form homo- or hetero-octamers (Tingey *et al.*, 1987; Dubois *et al.*, 1996) and various octet formations may account for the multi-faceted roles glutamine synthetase plays in overall nitrogen metabolism.

Plastidic and cytoplasmic glutamine synthetase genes have been studied in a number of plants and show a variety of regulation patterns (Lam *et al.*, 1996). Rice plants, which utilize a C3 carbon metabolism and are typically grown in water flooded soils, appear to express cytosolic forms of GS in the submerged root tissues. In the rice plant leaves, cytosolic forms are found in vascular tissues and a plastidic form shows light-regulated expression (see Tobin and Yamaya, 2001). In barley, which also utilizes a C3 carbon metabolism pathway but is grown in soil with good aeration, both plastid and cytosolic versions are found to be expressed in the root tissues (see Tobin and Yamaya, 2001). In pea, which fixes nitrogen via a symbiotic relationship with nitrogen fixing bacteria, only cytosolic forms were found in root tissues and, as found with other plants, the plastidic version is light regulated (Tingey *et al.*, 1988).

Promoters have been isolated for a number of GS genes and have been found to be diverse in sequence and activity. For example, glutamine synthetase  $\gamma$  and  $\beta$  from French bean are both expressed in the roots, yet the two genes showed different spatial and temporal patterns of expression (Forde *et al.*, 1989, 1990). Promoters for cytosolic (GS3A) and chloroplastic (GS2) from pea were isolated and joined to a reporter gene. The assay showed that the two promoters exhibited independent expression patterns which indicated non-redundant functions for these genes (Edwards *et al.*, 1990).

The cereal crop maize (*Zea mays* or corn) utilizes a C4 type metabolism for managing carbon resources. Li *et al.*, (1993) and Sakakibara *et al.*, (1992) reported that a total of six

different GS genes in maize showed five different patterns of transcript accumulation in a variety of plant tissues. Protein studies demonstrated that a pair of glutamine synthetase isozymes were expressed to high levels in kernels during development (Muhitch, 1988; 1989). Detailed studies with monoclonal antibodies distinguished between the isoforms and showed that while form GS<sub>p2</sub> was found the pedicel and other tissues, form GS<sub>p1</sub> was localized mainly to the pedicel (Muhitch *et al.*, 1995), a tissue that joins the developing kernel to the cob and houses the vascular tissue that feeds the developing kernel. Rastogi *et al.*, (1998) disclosed that the gene for pedicel specific GS<sub>p1</sub> was a previously identified cytosolic gene, GS<sub>1-2</sub>. Earlier research had reported that GS<sub>1-2</sub> RNA accumulated mainly in roots (Li *et al.*, 1993), but contradictory studies showed no accumulation of this RNA in roots (Sakakibara *et al.*, 1995). Later work demonstrated that GS<sub>1-2</sub> RNA was present in pedicel tissues and increased in accumulation from at least 7 to at least 33 days after pollination (Rastogi *et al.*, 1998).

Seed development in maize, and other crops, requires the transport and transfer of carbon, nitrogen and other nutrients from vegetative plant sources via the phloem, through the pedicel, to the seed, or kernel sink. Uptake of these nutrients is critical for proper kernel development, reducing kernel abortion, grain fill, grain quality and overall grain yield. Nitrogen is carried in the vascular sap in the form of amino acids, in particular, glutamine, glutamate, aspartate, alanine and serine (Lyznik *et al.*, 1982; Muhitch, 1989; 1995) with glutamine being one of the most abundant (Lyznik, *et al.*, 1982; Porter *et al.*, 1987; Oliveira *et al.*, 2001).

Nutrient molecules are unloaded from the pedicel vascular sap via parenchymal cells in the phloem and move through several layers of tissue including the pedicel-placento-chalazal region and the endosperm basal transfer cell layer as they cross from maternal tissue to developing endosperm and embryo (see, for example, Kiesselbach and Walker, 1952; Lyznik *et al.*, 1982; Thorne, 1985; Muhitch, 1993). The role of these tissues in metabolite transfer is very important as the developing kernel lacks vascular tissue of its own. Glutamine synthetase in the pedicel region is negligible very early after pollination, increases beginning about 10 days post-pollination (Muhitch, 1988) and activity increases with kernel development. Maximum glutamine synthetase activity, observed around 28 days post pollination, is coincident with maximum nitrogen

assimilation into the kernel followed by a decrease in activity as the kernel matures to completion (Muhitch, 1988; 1989). As the kernel increases in size and matures, movement of nutrient molecules into the kernel decreases significantly, and certain transfer tissue are eventually crushed, effectively sealing off the mature kernel from the parent plant tissue (Kiesselbach and Walker, 1952).

Although the above studies have provided a number of useful tools for the generation of transgenic plants, there is still a great need in the art for novel promoter sequences with beneficial expression characteristics, particularly for promoters which are developmentally regulated in tissues which affect kernel development. The number of effective promoters available for use with transgenes in maize is not abundant and those specific to kernel development even smaller. It would be especially advantageous to identify a promoter which plays a role in the import of nutrients into a developing seed as manipulations with such a promoter may allow for improvements to kernel development, grain yield, grain quality, pest resistance, stress resistance, fertility or decreased kernel abortion.

New promoters, such as that of the present invention, and especially promoters that will express differentially in maize female reproductive tissues, are useful. Such expression specific promoters are useful in minimizing yield drag and other potential adverse physiological effects on maize growth and development that might be encountered by high-level, non-inducible, constitutive expression of a transgenic protein in a plant. A wider range of effective promoters also may make it possible to introduce multiple transgenes into a plant, each fused to a different promoter, thereby minimizing the risk of DNA sequence homology dependent transgene inactivation (co-suppression). Therefore, there is a great need in the art for the identification of novel developmentally regulated, reproductive tissue specific promoters which can be used for the high-level expression of selected transgenes in economically important crop plants.

### **SUMMARY OF THE INVENTION**

In one aspect, the invention provides an isolated nucleic acid comprising a maize female reproductive tissue specific glutamine synthetase (GS<sub>1-2</sub>) promoter. Still further provided by the invention is a maize GS<sub>1-2</sub> promoter isolatable from the nucleic acid sequence of SEQ ID NO:18. In particular embodiments, a maize GS<sub>1-2</sub> promoter in accordance with the invention may  
5 comprise from about 135 to about 2547, about 250 to about 2547, about 400 to about 2547, about 750 to about 2547, about 1000 to about 2547, about 1500 to about 2547, about 1750 to about 2547, about 2000 to about 2547, about 2250 to about 2547, about 2500 to about 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18, as well as all lengths of contiguous nucleotides within such sizes. In further embodiments, the GS<sub>1-2</sub> promoter may  
10 comprise the nucleic acid sequence of SEQ ID NO:18.

An isolated nucleic acid comprising a maize GS<sub>1-2</sub> promoter in accordance with the invention may further comprising an enhancer, for example, an intron. In one embodiment, the intron is a rice actin 1 intron 1 or a rice actin 2 intron 1. The isolated nucleic acid may further  
15 comprise a 3' untranslated region (3' UTR), such as a *pinII* 3' UTR.

In another aspect, the invention provides a transgenic plant stably transformed with a selected DNA comprising a maize GS<sub>1-2</sub> promoter. In particular embodiments of the invention, the maize GS<sub>1-2</sub> promoter may comprise from about 135 to about 2547, about 250 to about 2547, about 400 to about 2547, about 750 to about 2547, about 1000 to about 2547, about 1500 to about 2547, about 1750 to about 2547, about 2000 to about 2547, about 2250 to about 2547, about 2500 to about 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. In one embodiment of the invention, the GS<sub>1-2</sub> promoter comprises the nucleic acid sequence of  
20 SEQ ID NO:18.

The selected DNA may further comprise any additional desired sequences. In one embodiment of the invention, the selected DNA further comprises a selected heterologous coding region operably linked to the maize GS<sub>1-2</sub> promoter. Potentially any coding sequence could be employed with the maize GS<sub>1-2</sub> promoter, including a selected coding region which encodes a  
30 protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral

disease resistance, nematode disease resistance, herbicide resistance, enhanced grain composition or quality, nutrient transporter functions, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics. Where the selected coding region encodes a protein imparting a selectable marker phenotype, the protein may be selected from, for example, the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase, anthranilate synthase and glyphosate oxidoreductase.

The selected coding region may be operably linked to a 3' untranslated region, for example, a *pinII* 3' UTR. Benefit may also be realized by including an enhancer with the selected DNA. Examples of such an enhancer include the rice actin 1 intron 1 and rice actin 2 intron 1.

The selected DNA may further comprise DNA from a cloning vector, such as plasmid DNA, or alternatively, may have been introduced as an expression cassette isolated from such vector DNA. The selected DNA may also comprise a sequence encoding a signal peptide. Examples of signal peptides that could be used include a peroxisomal targeting peptide or a chloroplast transit peptide. Examples of a chloroplast transit peptide include the group consisting of chlorophyll a/b binding protein transit peptide, small subunit of ribulose biphosphate carboxylase transit peptide, EPSPS transit peptide and dihydrodipicolinic acid synthase transit peptide.

A transgenic plant comprising a selected DNA in accordance with the invention may be potentially any type of plant, including a monocotyledonous or dicotyledonous plant. Examples of monocotyledonous plants include wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane. In one embodiment of the invention, the monocotyledonous plant is maize. Examples of dicotyledonous plants include tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton. In one embodiment of the invention the dicotyledonous plant is a soybean plant. The transgenic plant prepared in accordance with the invention may be of any

generation, including a fertile R<sub>0</sub> transgenic plant as well as seeds thereof, wherein the seed comprises the selected DNA. Also included within the invention are progeny plants of any generation such a fertile R<sub>0</sub> transgenic plant, wherein the progeny plant comprises said selected DNA, as well as seed of a progeny plant, wherein said seed comprises said selected DNA.

5

In yet another aspect, the invention provides a crossed fertile transgenic plant prepared according to the method comprising the steps of: (i) obtaining a fertile transgenic plant comprising a selected DNA comprising a maize GS<sub>1-2</sub> promoter, wherein the maize GS<sub>1-2</sub> promoter is isolatable from the nucleic acid sequence of SEQ ID NO:18; (ii) crossing the fertile transgenic  
10 plant with itself or with a second plant lacking said selected DNA to prepare the seed of a crossed fertile transgenic plant, wherein said seed comprises said selected DNA; and (iii) planting said seed to obtain a crossed fertile transgenic plant. The invention also includes a seed or seeds of such a crossed fertile transgenic plant, wherein said seed comprises said selected DNA. The  
15 crossed fertile transgenic plant may be potentially any type of plant, including a monocotyledonous or dicotyledonous plant. Examples of monocotyledonous plants include wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane. In one embodiment of the invention, the monocotyledonous plant is maize. Examples of dicotyledonous plants  
20 include tobacco, tomato, potato, soybean, cotton, canola, alfalfa, sunflower, and cotton. In one embodiment of the invention the dicotyledonous plant is a soybean plant. The selected DNA may have been inherited through a parent used a male or a female at any given generation. In one embodiment of the invention, the second plant is an inbred plant. Where the second plant is an inbred, the crossed fertile transgenic plant may be a hybrid, or also inbred where it is crossed with  
25 itself.

The crossed fertile transgenic plant may comprise any of the maize GS<sub>1-2</sub> promoter compositions provided by the invention. In one embodiment of the invention, the maize GS<sub>1-2</sub> promoter comprises from about 135 to about 2547, about 250 to about 2547, about 400 to about 2547, about 750 to about 2547, about 1000 to about 2547, about 1500 to about 2547, about 1750 to about 2547, about 2000 to about 2547, about 2250 to about 2547, about 2500 to about  
30 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. In another

25094423.1

embodiment of the invention, the fertile transgenic plant may comprise the full length of the nucleic acid sequence of SEQ ID NO:18, or a derivative thereof. The maize GS<sub>1-2</sub> promoter may further be operably linked to a selected heterologous coding region. Potentially any coding region could be used, including a selected coding region which encodes a protein selected from the group consisting of a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions; enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics.

The plant may further comprise a selected DNA comprising an enhancer, for example, a rice actin 1 intron 1 and rice actin 2 intron 1. The selected coding region may be operably linked to a 3' untranslated region, for example, a *pinII* 3' UTR.

In still yet another aspect, the invention provides a method of expressing a selected protein in a transgenic plant comprising the steps of: (i) obtaining a construct comprising a selected coding region operably linked to a maize GS<sub>1-2</sub> promoter, wherein the maize GS<sub>1-2</sub> promoter is isolatable from the nucleic acid sequence of SEQ ID NO:18; (ii) transforming a recipient plant cell with the construct; and (iii) regenerating a transgenic plant expressing the selected protein from the recipient plant cell. In one embodiment of the invention, the transgenic plant is fertile. The method may further comprise the step of obtaining seed from the fertile transgenic plant, and may still further comprise obtaining a progeny plant of any generation from the fertile transgenic plant. The transforming may be achieved in any manner, including a method selected from the group consisting of microprojectile bombardment, PEG mediated transformation of protoplasts, electroporation, silicon carbide fiber mediated transformation, or *Agrobacterium*-mediated transformation. In one embodiment of the invention, the step of transforming comprises microprojectile bombardment. The recipient plant cell may be of any type desired, including from a monocotyledonous or dicotyledonous plant. Examples of such a monocotyledonous plant include wheat, maize, rye, rice, turfgrass, oat, barley, sorghum, millet, and sugarcane. In one

embodiment of the invention, the monocotyledonous plant is a maize plant. Examples of dicotyledonous plants include tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton.

5 Any type of selected coding region may be used with the maize GS<sub>1-2</sub> promoter, including a coding region encoding a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female  
10 sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics. The construct used may further comprise any additional sequences desired, including an enhancer. Exemplary enhancers include the rice actin 1 intron 1 and rice actin 2 intron 1. The selected coding region may be operably linked to a 3' untranslated region, for example, a *pinII* 3' UTR.

15 In still yet another aspect, the invention provides a method of plant breeding comprising the steps of: (i) obtaining a transgenic plant comprising a selected DNA comprising a maize GS<sub>1-2</sub> promoter, wherein the maize GS<sub>1-2</sub> promoter is isolatable from the nucleic acid sequence of SEQ ID NO:18; and (ii) crossing the transgenic plant with itself or a second plant. The transgenic  
20 plant may be of potentially any species, including monocotyledonous or dicotyledonous plants. Examples of such monocotyledonous plants include wheat, maize, oat, barley, rye, rice, turfgrass, sorghum, millet and sugarcane. In one embodiment of the invention, the monocotyledonous plant is a maize plant. Examples of dicotyledonous plants include tobacco, tomato, potato, soybean, canola, sunflower, alfalfa and cotton. The selected DNA may comprise any of the maize GS<sub>1-2</sub>  
25 promoter compositions provided by the invention, and may comprise from about 135 to about 2547, about 250 to about 2547, about 400 to about 2547, about 750 to about 2547, about 1000 to about 2547, about 1500 to about 2547, about 1750 to about 2547, about 2000 to about 2547, about 2250 to about 2547, about 2500 to about 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. The maize GS<sub>1-2</sub> promoter may also comprise the nucleic acid  
30 sequence of SEQ ID NO:18, or a derivative thereof, such as a deletion mutant.



In one embodiment of the invention, the transgenic plant is crossed with the second plant. The second plant may be an inbred plant. The method may further comprise the steps of: (i) collecting seeds resulting from said crossing; (ii) growing said seeds to produce progeny plants; (iii) identifying a progeny plant comprising said selected DNA; and (iv) crossing said progeny plant with itself or a third plant. The progeny plant can inherit the selected DNA through a parent used as a male or female at any given generation. In one embodiment of the invention, the second plant and the third plant are of the same genotype, and further, may be inbred plants. The selected DNA may comprise potentially any coding region, for example, a coding region which encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance, herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics. The selected DNA may further comprise a genetic element which enhances the expression of the protein in the transgenic plant, including a rice actin 1 intron 1 and the rice actin 2 intron 1.

In still yet another aspect, the invention provides a transgenic plant cell stably transformed with a selected DNA comprising a maize GS<sub>1-2</sub> promoter, wherein said maize GS<sub>1-2</sub> promoter is isolatable from the nucleic acid sequence of SEQ ID NO:18. The plant cell may comprise any of the maize GS<sub>1-2</sub> promoter compositions provided by the invention, and may comprise from about 135 to about 2547, about 250 to about 2547, about 400 to about 2547, about 750 to about 2547, about 1000 to about 2547, about 1500 to about 2547, about 1750 to about 2547, about 2000 to about 2547, about 2250 to about 2547, about 2500 to about 2547 contiguous nucleotides of the nucleic acid sequence of SEQ ID NO:18. The maize GS<sub>1-2</sub> promoter may also comprise the nucleic acid sequence of SEQ ID NO:18, or a derivative thereof. The selected DNA may further comprise potentially any selected coding region operably linked to the maize GS<sub>1-2</sub> promoter, including a selected coding region which encodes a protein imparting insect resistance, bacterial disease resistance, fungal disease resistance, viral disease resistance, nematode disease resistance,

herbicide resistance, nutrient transporter functions, enhanced grain composition or quality, enhanced nutrient utilization, enhanced environment or stress resistance, reduced mycotoxin contamination, female sterility, a selectable marker phenotype, a screenable marker phenotype, a negative selectable marker phenotype, or altered plant agronomic characteristics. Where the selected coding sequence encodes a protein which imparts a selectable marker phenotype, exemplary coding sequences encode a protein selected from the group consisting of phosphinothricin acetyltransferase, glyphosate resistant EPSPS, aminoglycoside phosphotransferase, hygromycin phosphotransferase, neomycin phosphotransferase, dalapon dehalogenase, bromoxynil resistant nitrilase, anthranilate synthase and glyphosate oxidoreductase.

The selected coding region may be operably linked to a 3' UTR, such as a *pinII* 3' UTR. The selected DNA may also comprise an enhancer, including a rice actin 1 intron 1 and rice actin 2 intron 1.

The selected DNA may also comprise vector DNA, such as plasmid DNA, or may be isolated from such DNA. The selected DNA also may comprise a sequence encoding a signal peptide, including a peroxisomal targeting peptide or a chloroplast transit peptide. Exemplary transit peptides include a transit peptide selected from the group consisting of chlorophyll a/b binding protein transit peptide, small subunit of ribulose biphosphate carboxylase transit peptide, EPSPS transit peptide and dihydrodipicolinic acid synthase transit peptide. The transgenic plant cell may be of any species, and may be from a monocotyledonous or dicotyledonous plant. Exemplary monocotyledonous plants include wheat, maize, rye, rice, oat, barley, turfgrass, sorghum, millet and sugarcane. In one embodiment of the invention, the plant is a maize plant. Exemplary dicotyledonous plants include tobacco, tomato, potato, soybean, cotton, canola, alfalfa and sunflower. In one embodiment of the invention, the dicotyledonous plant is a soybean plant.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**FIG. 1: Primer alignment.** Location of the primers, relative to the maize GS<sub>1-2</sub> coding sequence, used for the inverse and confirmation PCR reactions.

**FIG. 2:** Sequence of the 2547 bp maize glutamine synthetase (GS<sub>1-2</sub>) promoter element (SEQ ID NO:18).

**FIG. 3:** Map of plasmid pMON65159. The plasmid comprises a 2547 base pair sequence comprising the maize glutamine synthase (GS<sub>1-2</sub>) promoter operably linked to a rice actin 1 intron 1, an *E. coli uidA* gene and 3' untranslated region and polyadenylation signal sequences derived from the potato *pinII* gene. The vector also comprises a Cauliflower Mosaic Virus 35S promoter operably linked to an hsp70 intron, a neomycin phosphotransferase II coding sequence, and 3' untranslated region and polyadenylation signal sequences derived from the nopaline synthase gene (*nos*).

**FIG. 4:** Map of plasmid pMON30113. The plasmid comprises an expression cassette containing a Cauliflower Mosaic Virus 35S promoter operably linked to an hsp70 intron, a neomycin phosphotransferase II coding sequence, and termination and polyadenylation signal sequences derived from the nopaline synthase gene. PMON30113 further contains an expression cassette comprising a Cauliflower Mosaic Virus 35S promoter operably linked to a rice actin 1 intron 1 sequence, a green fluorescent protein coding sequence, and termination and polyadenylation signal sequences derived from the hsp70 gene.

### **DETAILED DESCRIPTION OF THE INVENTION**

The utility of transgene expression in plants is a function of the transgene's promoter. The number of effective promoters available for use with transgenes in plants is limited. New promoters, especially promoters that will express differentially in plant tissues, such as those that are spatially and/or temporally expressed or are expressed in reproductive tissues involved in kernel development, would have significant utility in transgenic plants. Such expression specific promoters could be useful in minimizing yield drag and other potential adverse physiological effects on maize growth and development that might be encountered by constitutive expression of a particular transgenic proteins in maize plants.

The current invention overcomes deficiencies in the prior art by providing novel methods and compositions for the efficient expression of transgenes in plants, especially in the female reproductive tissues supporting the developing seed. In particular, the current invention provides a promoter of a *Zea mays* cytoplasmic pedicel-specific, glutamine synthetase (GS<sub>1-2</sub>) gene. The GS<sub>1-2</sub> promoter described herein represents a developmentally regulated promoter for which expression increases as the kernel develops to maturity. The promoter functions in tissues that are involved in the import of key nutrients to the developing seed. In addition, this promoter is also found to be active in the cob tissue and at the point of silk attachment to the kernel of unpollinated maize.

A pedicel-specific, developmentally regulated promoter such as maize GS<sub>1-2</sub> may find wide utility in directing the expression of potentially any gene which one desires to have expressed in tissue supporting the developing kernel. This promoter represents a significant advance in that it is capable of directing developmentally regulated expression of transgenes in tissues affecting import of nutrients into developing maize seed as well as in the pre- and post-pollination cob tissue. The specific nature of the promoter of the invention is important in that it allows expression of a transgene operatively linked to the promoter in specific tissues of the plant related to reproduction and the production of seed. By avoiding continuous high-level expression of transgenes and avoiding expression in non-target tissues, any undesired effects caused by continual overexpression of transgenes can be minimized or eliminated.

The GS<sub>1-2</sub> promoter sequence of the invention is exemplified by the nucleic acid sequence given in SEQ ID NO:18. However, in addition to the unmodified GS<sub>1-2</sub> promoter sequence of SEQ ID NO:18, the current invention includes derivatives of this sequence and compositions made therefrom. In particular, the present disclosure provides the teaching for one of skill in the art to make and use derivatives of the GS<sub>1-2</sub> promoter. For example, the disclosure provides the teaching for one of skill in the art to delimit the functional elements within the GS<sub>1-2</sub> promoter and to delete any non-essential elements. Functional elements also could be modified to increase the utility of the sequences of the invention for any particular application. For example, a functional region within the GS<sub>1-2</sub> promoter of the invention could be modified to cause or increase inducible

expression. Such changes could be made by site-specific mutagenesis techniques, for example, as described below.

One important application of the GS<sub>1-2</sub> promoter will be in the construction of vectors designed for introduction into plants by genetic transformation. By including an enhancer with the GS<sub>1-2</sub> promoter, such as an actin 1 intron 1 or actin 2 intron 1, one may potentially increase the level of expression of coding regions operably linked to the GS<sub>1-2</sub> promoter. It also is believed that benefit will be obtained by including a 3' untranslated region (3' UTR) and polyadenylation sequences with transgenes operably linked to the GS<sub>1-2</sub> promoter. One such 3' UTR that could be used is from a gene encoding nopaline synthase (*nos*) from *Agrobacterium tumefaciens* (Bevan *et al.*, 1983). Alternatively, one could utilize the *pinII* 3' UTR from potato (Graham *et al.*, 1986). A third possible 3' UTR that could be used is from a gene encoding the small subunit of a ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), and more specifically, from a rice Rubisco gene (PCT Publication WO 00/70066).

## **I. Derivatives of the Sequences of the Invention**

As indicated, an important aspect of the invention provides derivatives of the maize GS<sub>1-2</sub> promoter. In particular, the current invention includes sequences which have been derived from the maize GS<sub>1-2</sub> promoter disclosed herein. One efficient means for preparing such derivatives comprises introducing mutations into the sequences of the invention, for example, the sequence given in SEQ ID NO:18. Such mutants may potentially have enhanced or altered function relative to the native sequence or alternatively, may be silent with regard to function.

Mutagenesis may be carried out at random and the mutagenized sequences screened for function in a trial-by-error procedure. Alternatively, particular sequences which provide the GS<sub>1-2</sub> promoter with desirable expression characteristics could be identified and these or similar sequences introduced into other related or non-related sequences via mutation. Similarly, non-essential elements may be deleted without significantly altering the function of the elements. It

further is contemplated that one could mutagenize these sequences in order to enhance their utility in expressing transgenes in a particular species, for example, in maize.

The means for mutagenizing a DNA segment encoding a GS<sub>1-2</sub> promoter sequence of the current invention are well-known to those of skill in the art. Mutagenesis may be performed in accordance with any of the techniques known in the art, such as, but not limited to, synthesizing an oligonucleotide having one or more mutations within the sequence of a particular regulatory region. In particular, site-specific mutagenesis is a technique useful in the preparation of promoter mutants, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to about 75 nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double-stranded plasmids also are routinely employed in site-directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

Site-directed mutagenesis in accordance herewith typically is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector which includes within its sequence a DNA sequence which encodes the maize GS<sub>1-2</sub> promoter. An

oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector and subjected to DNA polymerizing enzymes such as the *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and cells are selected which include recombinant vectors bearing the mutated sequence arrangement. Vector DNA can then be isolated from these cells and used for plant transformation. A genetic selection scheme was devised by Kunkel *et al.* (1987) to enrich for clones incorporating mutagenic oligonucleotides. Alternatively, the use of PCR with commercially available thermostable enzymes such as *Taq* polymerase may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector. The PCR-mediated mutagenesis procedures of Tomic *et al.* (1990) and Upender *et al.* (1995) provide two examples of such protocols. A PCR employing a thermostable ligase in addition to a thermostable polymerase also may be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector.

The preparation of sequence variants of the selected promoter DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained. For example, recombinant vectors encoding the desired promoter sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

As used herein, the term “oligonucleotide-directed mutagenesis procedure” refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term “oligonucleotide directed mutagenesis procedure” also is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term “template-dependent

process" refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson and Ramstad, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U.S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety. A number of template dependent processes are available to amplify the target sequences of interest present in a sample, such methods being well known in the art and specifically disclosed herein below.

One efficient, targeted means for preparing mutagenized promoters or enhancers relies upon the identification of putative regulatory elements within the target sequence. This can be initiated by comparison with, for example, promoter sequences known to be expressed in a similar manner. Sequences which are shared among elements with similar functions or expression patterns are likely candidates for the binding of transcription factors and are thus likely elements which confer expression patterns. Confirmation of these putative regulatory elements can be achieved by deletion analysis of each putative regulatory region followed by functional analysis of each deletion construct by assay of a reporter gene which is functionally attached to each construct. As such, once a starting promoter or intron sequence is provided, any of a number of different functional deletion mutants of the starting sequence could be readily prepared.

As indicated above, deletion mutants of the GS<sub>1-2</sub> promoter also could be randomly prepared and then assayed. With this strategy, a series of constructs are prepared, each containing a different portion of the clone (a subclone), and these constructs are then screened for activity. A suitable means for screening for activity is to attach a deleted promoter construct to a selectable or screenable marker, and to isolate only those cells expressing the marker protein. In this way, a number of different, deleted promoter constructs are identified which still retain the desired, or even enhanced, activity. The smallest segment which is required for activity is thereby identified through comparison of the selected constructs. This segment may then be used for the construction of vectors for the expression of exogenous protein.



## **II. Plant Transformation Constructs**

The construction of vectors which may be employed in conjunction with plant transformation techniques according to the invention will be known to those of skill of the art in light of the present disclosure (see for example, Ausubel *et al.*, 2001; Sambrook and Russell, 2001; Gelvin *et al.*, 1990). The techniques of the current invention are thus not limited to any particular DNA sequences in conjunction with the GS<sub>1-2</sub> promoter of the invention. For example, the GS<sub>1-2</sub> promoter alone could be transformed into a plant with the goal of enhancing or altering the expression of one or more genes in the host genome.

One important use of the sequences of the invention will be in directing the expression of a selected coding region which encodes a particular protein or polypeptide product. The inventors also contemplate that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

In certain embodiments, the present inventors contemplate the transformation of a recipient cell with more than transformation construct. Two or more transgenes can be introduced in a single transformation event using either distinct selected-protein encoding vectors, or using a single vector incorporating two or more gene coding sequences. Of course, any two or more transgenes of any description, such as those conferring, for example, herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch quantity or properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

In other embodiments of the invention, it is contemplated that one may wish to employ replication-competent viral vectors for plant transformation. Such vectors include, for example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and pW1-GUS (Ugaki *et al.*, 1991).

These vectors are capable of autonomous replication in maize cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector also may be useful for delivery of genes flanked by DNA sequences from transposable elements such as Ac, Ds, or Mu. It has been proposed that transposition of these elements within the maize genome requires DNA replication (Laufs *et al.*, 1990). It also is contemplated that transposable elements would be useful for producing transgenic plants lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, *e.g.*, antibiotic resistance genes, or other selectable markers, and origins of DNA replication. It also is proposed that use of a transposable element such as Ac, Ds, or Mu would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells.

It further is contemplated that one may wish to co-transform plants or plant cells with two or more genes of interest. Co-transformation may be achieved using a vector containing the marker and another gene or genes of interest. Alternatively, different vectors, *e.g.*, plasmids, may contain the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. It is the present applicants' experience using microprojectile bombardment that a certain percentage of cells in which the marker has been introduced also have received the other gene(s) of interest, and that the selectable marker and gene(s) of interest are cointegrated at a single locus in the host genome. However, not all cells selected by means of the marker, will express the other proteins of interest which had been presented to the cells concurrently.

Vectors used for plant transformation may include, for example, plasmids, cosmids, YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), PACs (plant artificial chromosomes), or any other suitable cloning system. It is contemplated that utilization of cloning systems with large insert capacities will allow introduction of large DNA sequences comprising more than one selected gene. Introduction of such sequences may be facilitated by use of bacterial or yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes (PACs). For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Particularly useful for transformation are expression cassettes which have been isolated from such vectors. DNA segments used for transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduced into and have expressed in the host cells. These DNA segments can further include, in addition to a glutamine synthetase GS<sub>1-2</sub> promoter, structures such as promoters, enhancers, 3' untranslated regions, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction may encode a protein which will be expressed in the resultant recombinant cells resulting in a screenable or selectable trait and/or which will impart an improved phenotype to the resulting transgenic plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

#### **A. Regulatory Elements**

Constructs prepared in accordance with the current invention will include a maize GS<sub>1-2</sub> promoter or a derivative thereof. However, these sequences may be used in the preparation of transformation constructs which comprise a wide variety of other elements. One such application in accordance with the instant invention will be the preparation of transformation constructs comprising the GS<sub>1-2</sub> promoter operably linked to a selected coding region. By including an enhancer sequence with such constructs, the expression of the selected protein may be enhanced. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. In some instances, these 5' enhancing elements are introns. Deemed to be particularly useful as enhancers are the 5' introns of the rice actin 1 and rice actin 2 genes. Examples of other enhancers which could be used in accordance with the invention include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the maize alcohol dehydrogenase gene, the maize shrunken 1 gene and promoters from non-plant eukaryotes (*e.g.*, yeast; Ma *et al.*, 1988).

Where an enhancer is used in conjunction with a GS<sub>1-2</sub> promoter for the expression of a selected protein, it is believed that it will be preferred to place the enhancer between the promoter

and the start codon of the selected coding region. However, one also could use a different arrangement of the enhancer relative to other sequences and still realize the beneficial properties conferred by the enhancer. For example, the enhancer could be placed 5' of the promoter region, within the promoter region, within the coding sequence (including within any other intron sequences which may be present), or 3' of the coding region.

In addition to introns with enhancing activity, other types of elements can influence gene expression. For example, untranslated leader sequences predicted to enhance gene expression as well as "consensus" and preferred leader sequences have been made (Joshi, 1987). Preferred leader sequences are contemplated to include those which have sequences predicted to direct optimum expression of the attached coding region, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred, for example, sequences derived from the small subunit of ribulose biphosphate carboxylase (rubisco).

Specifically contemplated for use in accordance with the present invention are vectors which include the *ocs* enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (*ocs*) gene of *Agrobacterium* (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the *ocs* element and particularly multiple copies of the element, may be used to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation.

Ultimately, the most desirable DNA segments for introduction into a plant genome may be homologous genes or gene families which encode a desired trait, and which are introduced under the control of the maize GS<sub>1-2</sub> promoter. For example, it is envisioned that a particular use of the present invention may be the production of transformants comprising a transgene, the expression

of which is directed by the GS<sub>1-2</sub> promoter, whereby the expression is enhanced by an actin 1 intron 1 or actin 2 intron 1.

It also is contemplated that expression of one or more transgenes may be eliminated upon induction of the GS<sub>1-2</sub> promoter provided herein. In particular, by operably linking the GS<sub>1-2</sub> promoter to a particular coding sequence in antisense orientation, accumulation of the respective protein encoded by the sense transcript could be eliminated or decreased upon induction of the GS<sub>1-2</sub> promoter. This could allow, for example, inducible elimination of a particular gene product which would contribute to the ill effects of osmotic stress or attack by pests, disease, or other conditions.

It also is contemplated that it may be useful to target DNA within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Particular DNA sequences which are capable of targeting DNA to the nucleus are known, e.g., the *Agrobacterium tumefaciens* virD2 gene (Tinland *et al.*, 1995). Within the nucleus itself, it would be useful to target a gene in order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation replace an existing gene in the cell. Furthermore, it would be useful to target a transgene to integrate into the genome at a predetermined site from which it is known that gene expression occurs. Several site specific recombination systems exist which are known, including cre-lox (U.S. Patent No. 4,959,317) and FLP-FRT (U.S. Patent No. 5,527,695). Both of these cited site specific recombination systems have been shown to function in plants (Albert *et al.*, 1995; Lyznik *et al.*, 1996).

## **B. 3' Untranslated Regions**

Transformation constructs prepared in accordance with the invention will typically include a 3' end DNA sequence that acts as a signal to cease transcription and allow for the polyadenylation of the mRNA produced by coding sequences operably linked to a promoter. This 3' end sequence is often called a 3'UTR, 3' end or simply 3'. One type of 3'UTR sequence which may be used is a 3'UTR from the nopaline synthase gene of *Agrobacterium tumefaciens* (*nos* 3'

end; Bevan *et al.*, 1983). Where a 3' end other than a *nos* 3'UTR is used in accordance with the invention, the most preferred 3' ends are contemplated to be those from a gene encoding the small subunit of a ribulose-1,5-bisphosphate carboxylase-oxygenase (*rbcS*), and more specifically, from a rice *rbcS* gene (PCT Publication WO 00/70066), the 3'UTR for the T7 transcript of *Agrobacterium tumefaciens* (Dhaese *et al.*, 1983), the 3' end of the protease inhibitor I or II genes from potato (Graham *et al.*, 1986) or tomato (Pearce *et al.*, 1991), and the 3' region isolated from Cauliflower Mosaic Virus (Timmermans *et al.*, 1990). Alternatively, one also could use a gamma coixin, oleosin 3 or other 3'UTRs from the genus *Coix* (PCT Publication WO 99/58659).

### **C. Transit or Signal Peptides**

Sequences that are joined to the coding sequence of an expressed gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes, are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the endoplasmic reticulum, golgi apparatus, peroxisomes or glyoxysomes, and outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of a gene product protecting the protein from intracellular proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA 5' of the gene of interest may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It further is contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

A particular example of such a use concerns the direction of a protein conferring herbicide resistance, such as a mutant EPSPS protein, to a particular organelle such as the chloroplast

rather than to the cytoplasm. This is exemplified by the use of the *rbcs* transit peptide, the chloroplast transit peptide described in U.S. Patent No. 5,728,925, or the optimized transit peptide described in U.S. Patent No. 5,510,471, which confers plastid-specific targeting of proteins. In addition, it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole. A further use concerns the direction of enzymes involved in amino acid biosynthesis or oil synthesis to the plastid. Such enzymes include dihydrodipicolinic acid synthase which may contribute to increasing lysine content of a feed.

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. An intracellular targeting DNA sequence may be operably linked 5' or 3' to the coding sequence depending on the particular targeting sequence. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed.

#### D. Marker Genes

One application of the maize GS<sub>1-2</sub> promoter of the current invention will be in the expression of marker proteins. By employing a selectable or screenable marker gene as, or in addition to, the gene of interest, one can provide or enhance the ability to identify transformants.

5 "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can  
10 identify through observation or testing, *i.e.*, by "screening" (*e.g.*, the green fluorescent protein). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes also are genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include marker genes which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*,  $\alpha$ -amylase,  $\beta$ -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that  
25 becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall  
30 protein modified to include a unique epitope would satisfy all such requirements.



One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of maize HPRG (Steifel *et al.*, 1990) is preferred, as this molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller *et al.*, 1989) could be modified by the addition of an antigenic site to create a screenable marker.

One exemplary embodiment of a secretable screenable marker concerns the use of a HPRG sequence modified to include a 15 residue epitope from the pro-region of murine interleukin-1 $\beta$  (IL-1 $\beta$ ). However, virtually any detectable epitope may be employed in such embodiments, as selected from the extremely wide variety of antigen:antibody combinations known to those of skill in the art. The unique extracellular epitope, whether derived from IL-1 $\beta$  or any other protein or epitopic substance, can then be straightforwardly detected using antibody labeling in conjunction with chromogenic or fluorescent adjuncts.

#### 1. Selectable Markers

Many selectable marker coding regions may be used in connection with the GS<sub>1-2</sub> promoter of the present invention including, but not limited to, *neo* (Potrykus *et al.*, 1985) which provides kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; *bar*, which confers bialaphos or phosphinothricin resistance; a mutant EPSP synthase protein (Hinchee *et al.*, 1988) conferring glyphosate resistance; a nitrilase such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase (ALS) which confers resistance to imidazolinone, sulfonyleurea or other ALS inhibiting chemicals (EP 0 154 204); a methotrexate resistant DHFR (Thillet *et al.*, 1988), a dalapon dehalogenase that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase that confers resistance to 5-methyl tryptophan or other anthranilate synthase inhibiting compounds. Where a mutant EPSP synthase is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (U.S. Patent No.

5,188,642) or OTP (U.S. Patent No. 5,633,448) and use of a modified maize EPSPS (PCT Publication WO 97/04103).

An illustrative embodiment of selectable markers capable of being used in systems to select transformants are the enzyme phosphinothricin acetyltransferase, such as *bar* from *Streptomyces hygroscopicus* or *pat* from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.*, 1986; Twell *et al.*, 1989) causing rapid accumulation of ammonia and cell death.

Where one desires to employ bialaphos or phosphinothricin resistance in the practice of the invention, particularly useful genes for this purpose are the *bar* or *pat* genes obtainable from species of *Streptomyces* (e.g., ATCC No. 21,705). The cloning of the *bar* gene has been described (Murakami *et al.*, 1986; Thompson *et al.*, 1987) as has the use of the *bar* gene in the context of plants (De Block *et al.*, 1987; De Block *et al.*, 1989; U.S. Patent No. 5,550,318).

## 2. Screenable Markers

Screenable markers that may be employed include a  $\beta$ -glucuronidase or *uidA* gene (Jefferson *et al.*, 1986; the protein product is commonly referred to as GUS), isolated from *E. coli*, which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a  $\beta$ -lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a *xylE* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an  $\alpha$ -amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a  $\beta$ -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence

25094423.1

detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; PCT Publication WO 97/41228).

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding for the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which has the genotype r-g, b, Pl. Alternatively, any genotype of maize can be utilized if the C1 and R alleles are introduced together.

It further is proposed that R gene regulatory regions may be employed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe *et al.*, 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes for, *e.g.*, insect resistance, herbicide tolerance or other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (*e.g.*, P, S, Lc, *etc.*) in conjunction with the GS<sub>1-2</sub> promoter described herein. However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

Other screenable markers provide for visible light emission as a screenable phenotype. A screenable marker contemplated for use in the present invention is firefly luciferase, encoded by

25094423.1

the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein (GFP) is contemplated as a particularly useful reporter gene (Sheen *et al.*, 1995; Haseloff *et al.*, 1997; Reichel *et al.*, 1996; Tian *et al.*, 1997; PCT Publication WO 97/41228). Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light. Where use of a screenable marker gene such as *lux* or GFP is desired, the inventors contemplated that benefit may be realized by creating a gene fusion between the screenable marker gene and a selectable marker gene, for example, a GFP-NPTII gene fusion. This could allow, for example, selection of transformed cells followed by screening of transgenic plants or seeds. In a similar manner, it is possible to utilize other readily available fluorescent proteins such as red fluorescent protein (CLONTECH, Palo Alto, CA).

### **III. Exogenous Genes for Modification of Plant Phenotypes**

A particularly important advance of the present invention is that it provides methods and compositions for the efficient expression of selected genes in plant cells. In particular, the current invention provides a GS<sub>1-2</sub> promoter for the expression of selected proteins in plants. By including an enhancer with transformation constructs comprising the GS<sub>1-2</sub> promoter, increased expression of selected genes can be realized following introduction of the transformation construct into a host plant cell.

The choice of a selected gene for expression in a plant host cell in accordance with the invention will depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add commercially desirable, agronomically important or end-product traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance, insect resistance or tolerance, disease resistance or tolerance (viral, bacterial, fungal, nematode), stress tolerance and/or resistance, as exemplified by resistance or tolerance to

drought, heat, chilling, freezing, excessive moisture, salt stress and oxidative stress, increased yield, food or feed content and value, physical appearance, male sterility, drydown, standability, prolificacy, starch quantity and quality, oil quantity and quality, protein quality and quantity, amino acid composition, and the like.

5

In certain embodiments of the invention, transformation of a recipient cell may be carried out with more than one exogenous (selected) gene. As used herein, an “exogenous coding region” or “selected coding region” is a coding region not normally found in the host genome in an identical context. By this, it is meant that the coding region may be isolated from a different species than that of the host genome, or alternatively, isolated from the host genome, but is operably linked to one or more regulatory regions which differ from those found in the unaltered, native gene. Two or more exogenous coding regions also can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more coding sequences. For example, plasmids bearing a gene encoding phosphinothricin acetyltransferase (conferring resistance to glufosinate herbicide, *e.g.* *bar* or *pat*) and a gene encoding a glyphosate resistant EPSPS gene, *e.g.*, CP4 (U.S. Patent No. 5,627,061) in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect resistance gene, such as a Bt gene, along with a protease inhibitor gene such as *pinII*, or the use of *bar* in combination with either of the above genes. Of course, any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

25

#### **A. Herbicide Resistance**

The DNA segments encoding phosphinothricin acetyltransferase (*bar* and *pat*), EPSP synthase encoding genes conferring resistance to glyphosate, the glyphosate degradative enzyme gene *gox* encoding glyphosate oxidoreductase, *deh* (encoding a dehalogenase enzyme that inactivates dalapon), herbicide resistant (*e.g.*, sulfonylurea and imidazolinone) acetolactate synthase, and *bxn* genes (encoding a nitrilase enzyme that degrades bromoxynil) are examples of

30

25094423.1

herbicide resistant genes for use in transformation. The *bar* and *pat* genes code for an enzyme, phosphinothricin acetyltransferase (PAT), which inactivates the herbicide phosphinothricin and prevents this compound from inhibiting glutamine synthetase enzymes. The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP Synthase), is normally inhibited by the herbicide N-(phosphonomethyl)glycine (glyphosate) in plants and most microorganisms. However, genes are known that encode glyphosate-resistant EPSP synthase enzymes, including mutated EPSPS genes, *e.g.*, the *Salmonella typhimurium aroA* CT7 mutant (Comai *et al.*, 1985) and the naturally occurring glyphosate resistant EPSPS from *Agrobacterium*, CP4 (U.S. Patent No. 5,627,061). These genes are particularly contemplated for use in plant transformation. The *deh* gene encodes the enzyme dalapon dehalogenase and confers resistance to the herbicide dalapon (U.S. Patent No. 5,780,708). The *bxn* gene codes for a specific nitrilase enzyme that converts bromoxynil to a non-herbicidal degradation product.

#### **B. Insect Resistance**

Potential insect resistance genes that can be introduced include *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Bt genes may provide resistance to economically important lepidopteran or coleopteran pests such as European Corn Borer (ECB) and Western Corn Rootworm, respectively. It is contemplated that preferred Bt genes for use in the transformation protocols disclosed herein will be those in which the coding sequence has been modified to effect increased expression in plants, and more particularly, in maize. Means for preparing synthetic genes are well known in the art and are disclosed in, for example, U.S. Patent No. 5,500,365 and U.S. Patent No. 5,689,052, each of the disclosures of which are specifically incorporated herein by reference in their entirety. Examples of such modified Bt toxin genes include a synthetic Bt *CryIA(b)* gene (Perlak *et al.*, 1991), and the synthetic *CryIA(c)* gene termed 1800b (PCT Publication WO 95/06128). Some examples of other Bt toxin genes known to those of skill in the art are given in Table 1 below.

**Table 1: *Bacillus thuringiensis*  $\delta$ -Endotoxin Genes<sup>a</sup>**

<b>New Nomenclature</b>	<b>Old Nomenclature</b>	<b>GenBank Accession</b>
Cry1Aa	CryIA(a)	M11250
Cry1Ab	CryIA(b)	M13898
Cry1Ac	CryIA(c)	M11068
Cry1Ad	CryIA(d)	M73250
Cry1Ae	CryIA(e)	M65252
Cry1Ba	CryIB	X06711
Cry1Bb	ET5	L32020
Cry1Bc	PEG5	Z46442
Cry1Bd	CryE1	U70726
Cry1Ca	CryIC	X07518
Cry1Cb	CryIC(b)	M97880
Cry1Da	CryID	X54160
Cry1Db	PrtB	Z22511
Cry1Ea	CryIE	X53985
Cry1Eb	CryIE(b)	M73253
Cry1Fa	CryIF	M63897
Cry1Fb	PrtD	Z22512
Cry1Ga	PrtA	Z22510
Cry1Gb	CryH2	U70725
Cry1Ha	PrtC	Z22513
Cry1Hb		U35780
Cry1Ia	CryV	X62821
Cry1Ib	CryV	U07642
Cry1Ja	ET4	L32019
Cry1Jb	ET1	U31527
Cry1K		U28801
Cry2Aa	CryIIA	M31738
Cry2Ab	CryIIB	M23724
Cry2Ac	CryIIC	X57252
Cry3A	CryIIIA	M22472
Cry3Ba	CryIIIB	X17123
Cry3Bb	CryIIIB2	M89794
Cry3C	CryIIID	X59797
Cry4A	CryIVA	Y00423
Cry4B	CryIVB	X07423
Cry5Aa	CryVA(a)	L07025
Cry5Ab	CryVA(b)	L07026
Cry6A	CryVIA	L07022
Cry6B	CryVIB	L07024
Cry7Aa	CryIIIC	M64478
Cry7Ab	CryIIICb	U04367

New Nomenclature	Old Nomenclature	GenBank Accession
Cry8A	CryIIIE	U04364
Cry8B	CryIIIG	U04365
Cry8C	CryIIIF	U04366
Cry9A	CryIG	X58120
Cry9B	CryIX	X75019
Cry9C	CryIH	Z37527
Cry10A	CryIVC	M12662
Cry11A	CryIVD	M31737
Cry11B	Jeg80	X86902
Cry12A	CryVB	L07027
Cry13A	CryVC	L07023
Cry14A	CryVD	U13955
Cry15A	34kDa	M76442
Cry16A	cbm71	X94146
Cry17A	cbm71	X99478
Cry18A	CryBP1	X99049
Cry19A	Jeg65	Y08920
Cyt1Aa	CytA	X03182
Cyt1Ab	CytM	X98793
Cyt2A	CytB	Z14147
Cyt2B	CytB	U52043

<sup>a</sup>Adapted from: [http://epunix.biols.susx.ac.uk/Home/Neil\\_Crickmore/Bt/index.html](http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html)

Protease inhibitors also may provide insect resistance (Johnson *et al.*, 1989), and thus will have utility in plant transformation. The use of a protease inhibitor II gene, *pinII*, from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a *pinII* gene in combination with a Bt toxin gene, the combined effect of which has been discovered to produce synergistic insecticidal activity. Other genes which encode inhibitors of the insect's digestive system, or those that encode enzymes or co-factors that facilitate the production of inhibitors, also may be useful. This group may be exemplified by oryzacystatin and amylase inhibitors such as those from wheat and barley.

Also, genes encoding lectins may confer additional or alternative insecticide properties. Lectins (originally termed phytohemagglutinins) are multivalent carbohydrate-binding proteins which have the ability to agglutinate red blood cells from a range of species. Lectins have been identified recently as insecticidal agents with activity against weevils, ECB and rootworm



(Murdock *et al.*, 1990; Czapla & Lang, 1990). Lectin genes contemplated to be useful include, for example, barley and wheat germ agglutinin (WGA) and rice lectins (Gatehouse *et al.*, 1984), with WGA being preferred.

5 Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as, *e.g.*, lytic peptides, peptide hormones and toxins and venoms, form another aspect of the invention. For example, it is contemplated that the expression of juvenile hormone esterase, directed towards specific insect pests, also may result in insecticidal activity, or perhaps cause cessation of metamorphosis (Hammock *et al.*, 1990).

10 Transgenic plants expressing genes which encode enzymes that affect the integrity of the insect cuticle form yet another aspect of the invention. Such genes include those encoding, *e.g.*, chitinase, proteases, lipases and also genes for the production of nikkomycin, a compound that inhibits chitin synthesis, the introduction of any of which is contemplated to produce insect resistant plants. Genes that code for activities that affect insect molting, such as those affecting the production of ecdysteroid UDP-glucosyl transferase, also fall within the scope of the useful transgenes of the present invention.

15 Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests also are encompassed by the present invention. It may be possible, for instance, to confer insecticidal activity on a plant by altering its sterol composition. Sterols are obtained by insects from their diet and are used for hormone synthesis and membrane stability. Therefore, alterations in plant sterol composition by expression of novel genes, *e.g.*, those that directly promote the production of undesirable sterols or those that convert  
20 desirable sterols into undesirable forms, could have a negative effect on insect growth and/or development and hence endow the plant with insecticidal activity. Lipxygenases are naturally occurring plant enzymes that have been shown to exhibit anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Therefore, further embodiments of the invention concern transgenic plants with enhanced lipxygenase activity which may be resistant to insect  
25 feeding.  
30

*Tripsacum dactyloides* is a species of grass that is resistant to certain insects, including corn root worm. It is anticipated that genes encoding proteins that are toxic to insects or are involved in the biosynthesis of compounds toxic to insects will be isolated from *Tripsacum* and that these novel genes will be useful in conferring resistance to insects. It is known that the basis of insect resistance in *Tripsacum* is genetic, because said resistance has been transferred to *Zea mays* via sexual crosses (Branson and Guss, 1972). It further is anticipated that other cereal, monocot or dicot plant species may have genes encoding proteins that are toxic to insects which would be useful for producing insect resistant corn plants.

Further genes encoding proteins characterized as having potential insecticidal activity also may be used as transgenes in accordance herewith. Such genes include, for example, the cowpea trypsin inhibitor (CpTI; Hilder *et al.*, 1987) which may be used as a rootworm deterrent; genes encoding avermectin (Campbell, 1989; Ikeda *et al.*, 1987) which may prove particularly useful as a corn rootworm deterrent; ribosome inactivating protein genes; and even genes that regulate plant structures. Transgenic maize including anti-insect antibody genes and genes that code for enzymes that can convert a non-toxic insecticide (pro-insecticide) applied to the outside of the plant into an insecticide inside the plant also are contemplated.

### **C. Environment or Stress Resistance**

Improvement of a plants ability to respond to various environmental signals, such as but not limited to, light, CO<sub>2</sub> or nitrogen, or to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative stress, also can be effected through expression of novel genes.

It is proposed that benefits may be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein such as that of the Winter Flounder (Cutler *et al.*, 1989) or synthetic gene derivatives thereof. Improved chilling tolerance also may be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Wolter *et al.*, 1992). Resistance to oxidative stress (often exacerbated by conditions

such as chilling temperatures in combination with high light intensities) can be conferred by expression of superoxide dismutase (Gupta *et al.*, 1993), and may be improved by glutathione reductase (Bowler *et al.*, 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity zones.

It is proposed that expression of a gene encoding hemoglobin may enhance a plant's ability to assimilate and utilize oxygen, resulting in quicker germination, faster growing or maturing crops, or higher crop yields (Holmberg *et al.* 1997).

It is contemplated that the expression of novel genes that favorably affect plant water content, total water potential, osmotic potential, and turgor will enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in water availability, as compared to normal circumstances, and the ability of the plant to function and survive in lower-water environments. It is contemplated that drought resistance and/or drought tolerance may lead to increased yield under conditions of limited water availability. Alternatively, substantially consistent yields under conditions of adequate or limited water availability may be provided, i.e., yield stability. In this aspect of the invention it is proposed, for example, that the expression of genes encoding for the biosynthesis of osmotically-active solutes, such as polyol compounds, may impart protection against drought. Within this class are genes encoding for mannitol-L-phosphate dehydrogenase (Lee and Saier, 1983), trehalose-6-phosphate synthase (Kaasen *et al.*, 1992), and myo-inositol O-methyl transferase (U.S. Patent No. 5,563,324). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these introduced genes will result in the accumulation of either mannitol or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski *et al.*, 1992, 1993). Altered

water utilization in transgenic corn producing mannitol also has been demonstrated (U.S. Patent No. 5,780,709).

Similarly, the efficacy of other metabolites in protecting either enzyme function (*e.g.*, alanopine or propionic acid) or membrane integrity (*e.g.*, alanopine) has been documented (Loomis *et al.*, 1989), and therefore expression of genes encoding for the biosynthesis of these compounds might confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include fructose, erythritol (Coxson *et al.*, 1992), sorbitol, dulcitol (Karsten *et al.*, 1992), glucosylglycerol (Reed *et al.*, 1984; Erdmann *et al.*, 1992), sucrose, stachyose (Koster and Leopold, 1988; Blackman *et al.*, 1992), raffinose (Bernal-Lugo and Leopold, 1992), proline (Rensburg *et al.*, 1993), glycine betaine, ononitol and pinitol (Vernon and Bohnert, 1992). Continued canopy growth and increased reproductive fitness during times of stress will be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above and other such compounds. Currently preferred genes which promote the synthesis of an osmotically active polyol compound are genes which encode the enzymes mannitol-1-phosphate dehydrogenase, trehalose-6-phosphate synthase and myoinositol O-methyltransferase.

It is contemplated that the expression of specific proteins also may increase drought tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure *et al.*, 1989). All three classes of LEAs have been demonstrated in maturing (*i.e.*, desiccating) seeds. Within these 3 types of LEA proteins, the Type-II (dehydrin-type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (*i.e.*, Mundy and Chua, 1988; Piatkowski *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and drought tolerance (Fitzpatrick, 1993). In rice, expression of the HVA-1 gene influenced tolerance to water deficit and salinity (Xu *et al.*, 1996). Expression of structural genes from all three LEA groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero *et*

*al.*, 1990), which may confer various protective and/or repair-type functions during drought stress. It also is contemplated that genes that effect lipid biosynthesis and hence membrane composition might also be useful in conferring drought resistance on the plant.

5 Many of these genes for improving drought resistance have complementary modes of action. Thus, it is envisaged that combinations of these genes might have additive and/or synergistic effects in improving drought resistance in crop plants such as, for example, corn, soybean, cotton, or wheat. Many of these genes also improve freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be  
10 mitigated in similar fashion. Benefit may be conferred via constitutive expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero *et al.*, 1990 and Shagan *et al.*, 1993, which are incorporated herein by reference) or an ABA-inducible promoter such as the promoter of the present invention. Inducible, spatial and temporal  
15 expression patterns of these genes may enable plants to better withstand stress.

It is proposed that expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example, introduction and expression of genes that alter root characteristics may enhance water uptake. It  
20 also is contemplated that expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of genes that improve the synchrony of pollen shed and receptiveness of the female flower parts, *i.e.*, silks, would be of benefit. In addition it is proposed that expression of genes that minimize kernel abortion during times of stress would increase the amount of grain to be harvested and hence be of value.

25 Given the overall role of water in determining yield, it is contemplated that enabling corn and other crop plants to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of plants to maximize water usage across a full

range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

#### **D. Disease Resistance**

It is proposed that increased resistance to diseases may be realized through introduction of genes into plants, for example, into monocotyledonous plants such as maize. It is possible to produce resistance to diseases caused by viruses, bacteria, fungi and nematodes. It also is contemplated that control of mycotoxin producing organisms may be realized through expression of introduced genes.

Resistance to viruses may be produced through expression of novel genes. For example, it has been demonstrated that expression of a viral coat protein in a transgenic plant can impart resistance to infection of the plant by that virus and perhaps other closely related viruses (Cuozzo *et al.*, 1988, Hemenway *et al.*, 1988, Abel *et al.*, 1986). It is contemplated that expression of antisense genes targeted at essential viral functions also may impart resistance to viruses. For example, an antisense gene targeted at the gene responsible for replication of viral nucleic acid may inhibit replication and lead to resistance to the virus. It is believed that interference with other viral functions through the use of antisense genes also may increase resistance to viruses. . Similarly, ribozymes could be used in this context. Further, it is proposed that it may be possible to achieve resistance to viruses through other approaches, including, but not limited to the use of satellite viruses. Examples of viral and viral-like diseases, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Table 2.

**Table 2: Plant Virus and Virus-like Diseases**

<b>DISEASE</b>	<b>CAUSATIVE AGENT</b>
American wheat striate (wheat striate mosaic)	American wheat striate mosaic virus mosaic (AWSMV)
Barley stripe mosaic	Barley stripe mosaic virus (BSMV)
Barley yellow dwarf	Barley yellow dwarf virus (BYDV)
Brome mosaic	Brome mosaic virus (BMV)
Cereal chlorotic mottle*	Cereal chlorotic mottle virus (CCMV)
Corn chlorotic vein banding (Brazilian maize mosaic) <sup>1</sup>	Corn chlorotic vein banding virus (CCVBV)
Corn lethal necrosis	Virus complex (Maize chlorotic mottle virus(MCMV) and Maize dwarf mosaic virus (MDMV) A or B or Wheat streak mosaic virus(WSMV))
Cucumber mosaic	Cucumber mosaic virus (CMV)
Cynodon chlorotic streak*, <sup>1</sup>	Cynodon chlorotic streak virus (CCSV)
Johnsongrass mosaic	Johnsongrass mosaic virus (JGMV)
Maize bushy stunt	Mycoplasma-like organism (MLO) associated
Maize chlorotic dwarf	Maize chlorotic dwarf virus (MCDV)
Maize chlorotic mottle	Maize chlorotic mottle virus (MCMV)
Maize dwarf mosaic	Maize dwarf mosaic virus (MDMV) strains A, D, E and F
Maize leaf fleck	Maize leaf fleck virus (MLFV)
Maize line*	Maize line virus (MLV)
Maize mosaic (corn leaf stripe, enanismo rayado)	Maize mosaic virus (MMV)
Maize mottle and chlorotic stunt <sup>1</sup>	Maize mottle and chlorotic stunt virus*
Maize pellucid ringspot*	Maize pellucid ringspot virus (MPRV)
Maize raya gruesa*, <sup>1</sup>	Maize raya gruesa virus (MRGV)
maize rayado fino* (fine striping disease)	Maize rayado fino virus (MRFV)
Maize red leaf and red stripe*	Mollicute?
Maize red stripe*	Maize red stripe virus (MRSV)
Maize ring mottle*	Maize ring mottle virus (MRMV)
Maize rio IV*	Maize rio cuarto virus (MRCV)
Maize rough dwarf* (nanismo ruvido)	Maize rough dwarf virus (MRDV) (= Cereal tillering disease virus*)
Maize sterile stunt*	Maize sterile stunt virus (strains of barley yellow striate virus)
Maize streak*	Maize streak virus (MSV)
Maize stripe (maize chlorotic stripe, maize hoja blanca)	Maize stripe virus
Maize stunting*, <sup>1</sup>	Maize stunting virus
Maize tassel abortion*	Maize tassel abortion virus (MTAV)
Maize vein enation*	Maize vein enation virus (MVEV)

DISEASE	CAUSATIVE AGENT
Maize wallaby ear*	Maize wallaby ear virus (MWEV)
Maize white leaf*	Maize white leaf virus
Maize white line mosaic	Maize white line mosaic virus (MWLMV)
Millet red leaf*	Millet red leaf virus (MRLV)
Northern cereal mosaic*	Northern cereal mosaic virus (NCMV)
Oat pseudorosette* (zakuklivanie)	Oat pseudorosette virus
Oat sterile dwarf*	Oat sterile dwarf virus (OSDV)
Rice black-streaked dwarf*	Rice black-streaked dwarf virus (RBSDV)
Rice stripe*	Rice stripe virus (RSV)
Sorghum mosaic	Sorghum mosaic virus (SrMV), formerly sugarcane mosaic virus (SCMV) strains H, I and M
Sugarcane Fiji disease*	Sugarcane Fiji disease virus (FDV)
Sugarcane mosaic	Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B)
Vein enation*, <sup>1</sup>	Virus ?
Wheat spot mosaic <sup>1</sup>	Wheat spot mosaic virus (WSMV)

\*Not known to occur naturally on corn in the United States. <sup>1</sup> Minor viral disease.

It is proposed that increased resistance to diseases caused by bacteria and fungi also may be realized through introduction of novel genes. It is contemplated that genes encoding so-called "peptide antibiotics," pathogenesis related (PR) proteins, toxin resistance, and proteins affecting host-pathogen interactions such as morphological characteristics will be useful. Peptide antibiotics are polypeptide sequences which are inhibitory to growth of bacteria and other microorganisms. For example, the classes of peptides referred to as cecropins and magainins inhibit growth of many species of bacteria and fungi. It is proposed that expression of PR proteins in monocotyledonous plants such as maize may be useful in conferring resistance to bacterial disease. These genes are induced following pathogen attack on a host plant and have been divided into at least five classes of proteins (Bol *et al.*, 1990). Included amongst the PR proteins are  $\beta$ -1, 3-glucanases, chitinases, and osmotin and other proteins that are believed to function in plant resistance to disease organisms. Other genes have been identified that have antifungal properties, *e.g.*, UDA (stinging nettle lectin), hevein (Brookaert *et al.*, 1989; Barkai-Golan *et al.*, 1978), and *sor1* conferring resistance to photosensitizing toxins (Ehrenshaft *et al.*, 1999). It is known that certain plant diseases are caused by the production of phytotoxins. It is



proposed that resistance to these diseases would be achieved through expression of a novel gene that encodes an enzyme capable of degrading or otherwise inactivating the phytotoxin. It also is contemplated that expression of novel genes that alter the interactions between the host plant and pathogen may be useful in reducing the ability of the disease organism to invade the tissues of the host plant, e.g., an increase in the waxiness of the leaf cuticle or other morphological characteristics. Examples of bacterial and fungal diseases, including downy mildews, for which one could introduce resistance to in a transgenic plant in accordance with the instant invention, are listed below, in Tables 3, 4 and 5.

**Table 3: Plant Bacterial Diseases**

DISEASE	CAUSATIVE AGENT
Bacterial leaf blight and stalk rot	<i>Pseudomonas avenae</i> subsp. <i>avenae</i>
Bacterial leaf spot	<i>Xanthomonas campestris</i> pv. <i>holcicola</i>
Bacterial stalk rot	<i>Enterobacter dissolvens</i> = <i>Erwinia dissolvens</i>
Bacterial stalk and top rot	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> , <i>Erwinia chrysanthemi</i> pv. <i>zeae</i>
Bacterial stripe	<i>Pseudomonas andropogonis</i>
Chocolate spot	<i>Pseudomonas syringae</i> pv. <i>coronafaciens</i>
Goss's bacterial wilt and blight (leaf freckles and wilt)	<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i> = <i>Corynebacterium michiganense</i> pv. <i>nebraskense</i>
Holcus spot	<i>Pseudomonas syringae</i> pv. <i>syringae</i>
Purple leaf sheath	Hemiparasitic bacteria + (See under Fungi)
Seed rot-seedling blight	<i>Bacillus subtilis</i>
Stewart's disease (bacterial wilt)	<i>Pantoea stewartii</i> = <i>Erwinia stewartii</i>
Corn stunt (achapparramiento, maize stunt, Mesa Central or Rio Grande maize stunt)	<i>Spiroplasma kunkelii</i>

**Table 4: Plant Fungal Diseases**

DISEASE	PATHOGEN
Anthracnose leaf blight and anthracnose stalk rot	<i>Colletotrichum graminicola</i> (teleomorph: <i>Glomerella graminicola</i> Politis), <i>Glomerella tucumanensis</i> (anamorph: <i>Glomerella falcatum</i> Went)
Aspergillus ear and kernel rot	<i>Aspergillus flavus</i> Link:Fr.
Banded leaf and sheath spot*	<i>Rhizoctonia solani</i> Kühn = <i>Rhizoctonia microsclerotia</i> J. Matz (teleomorph: <i>Thanatephorus cucumeris</i> )
Black bundle disease	<i>Acremonium strictum</i> W. Gams = <i>Cephalosporium acremonium</i> Auct. non Corda

DISEASE	PATHOGEN
Black kernel rot*	<i>Lasiodiplodia theobromae</i> = <i>Botryodiplodia theobromae</i>
Borde blanco*	<i>Marasmiellus</i> sp.
Brown spot (black spot, stalk rot)	<i>Physoderma maydis</i>
Cephalosporium kernel rot	<i>Acremonium strictum</i> = <i>Cephalosporium acremonium</i>
Charcoal rot	<i>Macrophomina phaseolina</i>
Corticium ear rot*	<i>Thanatephorus cucumeris</i> = <i>Corticium sasakii</i>
Curvularia leaf spot	<i>Curvularia clavata</i> , <i>C. eragrostidis</i> , = <i>C. maculans</i> (teleomorph: <i>Cochliobolus eragrostidis</i> ), <i>Curvularia inaequalis</i> , <i>C. intermedia</i> (teleomorph: <i>Cochliobolus intermedius</i> ), <i>Curvularia lunata</i> (teleomorph: <i>Cochliobolus lunatus</i> ), <i>Curvularia pallescens</i> (teleomorph: <i>Cochliobolus pallescens</i> ), <i>Curvularia senegalensis</i> , <i>C. tuberculata</i> (teleomorph: <i>Cochliobolus tuberculatus</i> )
Didymella leaf spot*	<i>Didymella exitalis</i>
Diplodia ear rot and stalk rot	<i>Diplodia frumenti</i> (teleomorph: <i>Botryosphaeria festucae</i> )
Diplodia ear rot, stalk rot, seed rot and seedling blight	<i>Diplodia maydis</i> = <i>Stenocarpella maydis</i>
Diplodia leaf spot or leaf streak	<i>Stenocarpella macrospora</i> = <i>Diplodia macrospora</i>
*Not known to occur naturally on corn in the United States.	

**Table 5: Plant Downy Mildews**

DISEASE	CAUSATIVE AGENT
Brown stripe downy mildew*	<i>Sclerophthora rayssiae</i> var. <i>zeae</i>
Crazy top downy mildew	<i>Sclerophthora macrospora</i> = <i>Sclerospora macrospora</i>
Green ear downy mildew (graminicola downy mildew)	<i>Sclerospora graminicola</i>
Java downy mildew*	<i>Peronosclerospora maydis</i> = <i>Sclerospora maydis</i>
Philippine downy mildew*	<i>Peronosclerospora philippinensis</i> = <i>Sclerospora philippinensis</i>
Sorghum downy mildew	<i>Peronosclerospora sorghi</i> = <i>Sclerospora sorghi</i>
Spontaneum downy mildew*	<i>Peronosclerospora spontanea</i> = <i>Sclerospora spontanea</i>
Sugarcane downy mildew*	<i>Peronosclerospora sacchari</i> = <i>Sclerospora sacchari</i>

DISEASE	CAUSATIVE AGENT
Dry ear rot (cob, kernel and stalk rot)	<i>Nigrospora oryzae</i> (teleomorph: <i>Khuskia oryzae</i> )
Ear rots, minor	<i>Alternaria alternata</i> = <i>A. tenuis</i> , <i>Aspergillus glaucus</i> , <i>A. niger</i> , <i>Aspergillus</i> spp., <i>Botrytis cinerea</i> (teleomorph: <i>Botryotinia fuckeliana</i> ), <i>Cunninghamella</i> sp., <i>Curvularia pallescens</i> , <i>Doratomyces stemonitis</i> = <i>Cephalotrichum stemonitis</i> , <i>Fusarium culmorum</i> , <i>Gonatobotrys simplex</i> , <i>Pithomyces maydis</i> , <i>Rhizopus microsporus</i> Tiegh., <i>R. stolonifer</i> = <i>R. nigricans</i> , <i>Scopulariopsis brumptii</i> .
Ergot* (horse's tooth, diente de caballo)	<i>Claviceps gigantea</i> (anamorph: <i>Sphacelia</i> sp.)
Eyespot	<i>Aureobasidium zeae</i> = <i>Kabatella zeae</i>
Fusarium ear and stalk rot	<i>Fusarium subglutinans</i> = <i>F. moniliforme</i> var. <i>subglutinans</i>
Fusarium kernel, root and stalk rot, seed rot and seedling blight	<i>Fusarium moniliforme</i> (teleomorph: <i>Gibberella fujikuroi</i> )
Fusarium stalk rot, seedling root rot	<i>Fusarium avenaceum</i> (teleomorph: <i>Gibberella avenacea</i> )
Gibberella ear and stalk rot	<i>Gibberella zeae</i> (anamorph: <i>Fusarium graminearum</i> )
Gray ear rot	<i>Botryosphaeria zeae</i> = <i>Physalospora zeae</i> (anamorph: <i>Macrophoma zeae</i> )
Gray leaf spot (Cercospora leaf spot)	<i>Cercospora sorghi</i> = <i>C. sorghi</i> var. <i>maydis</i> , <i>C. zeae-maydis</i>
Helminthosporium root rot	<i>Exserohilum pedicellatum</i> = <i>Helminthosporium pedicellatum</i> (teleomorph: <i>Setosphaeria pedicellata</i> )
Hormodendrum ear rot (Cladosporium rot)	<i>Cladosporium cladosporioides</i> = <i>Hormodendrum cladosporioides</i> , <i>C. herbarum</i> (teleomorph: <i>Mycosphaerella tassiana</i> )
Hyalothyridium leaf spot*	<i>Hyalothyridium maydis</i>
Late wilt*	<i>Cephalosporium maydis</i>
Leaf spots, minor	<i>Alternaria alternata</i> , <i>Ascochyta maydis</i> , <i>A. tritici</i> , <i>A. zeicola</i> , <i>Bipolaris victoriae</i> = <i>Helminthosporium victoriae</i> (teleomorph: <i>Cochliobolus victoriae</i> ), <i>C. sativus</i> (anamorph: <i>Bipolaris sorokiniana</i> = <i>H. sorokinianum</i> = <i>H. sativum</i> ), <i>Epicoccum nigrum</i> , <i>Exserohilum prolatum</i> = <i>Drechslera prolatum</i> (teleomorph: <i>Setosphaeria prolata</i> ) <i>Graphium penicillioides</i> , <i>Leptosphaeria maydis</i> , <i>Leptothyrium zeae</i> , <i>Ophiosphaerella herpotricha</i> , (anamorph:

DISEASE	CAUSATIVE AGENT
Northern corn leaf blight (white blast, crown stalk rot, stripe)	<i>Scolecosporella</i> sp.), <i>Paraphaeosphaeria michotii</i> , <i>Phoma</i> sp., <i>Septoria zeae</i> , <i>S. zeicola</i> , <i>S. zeina</i>
Northern corn leaf spot, <i>Helminthosporium</i> ear rot (race 1)	<i>Setosphaeria turcica</i> (anamorph: <i>Exserohilum turcicum</i> = <i>Helminthosporium turcicum</i> )
Penicillium ear rot (blue eye, blue mold)	<i>Cochliobolus carbonum</i> (anamorph: <i>Bipolaris zeicola</i> = <i>Helminthosporium carbonum</i> )
Phaeocystostroma stalk rot and root rot	<i>Penicillium</i> spp., <i>P. chrysogenum</i> , <i>P. expansum</i> , <i>P. oxalicum</i>
Phaeosphaeria leaf spot*	<i>Phaeocystostroma ambiguum</i> , =
Physalospora ear rot ( <i>Botryosphaeria</i> ear rot)	<i>Phaeocystosporella zeae</i>
Purple leaf sheath	<i>Phaeosphaeria maydis</i> = <i>Sphaerulina maydis</i>
Pyrenochaeta stalk rot and root rot	<i>Botryosphaeria festucae</i> = <i>Physalospora zeicola</i> (anamorph: <i>Diplodia frumenti</i> )
Pythium root rot	Hemiparasitic bacteria and fungi
Pythium stalk rot	<i>Phoma terrestris</i> = <i>Pyrenochaeta terrestris</i>
Red kernel disease (ear mold, leaf and seed rot)	<i>Pythium</i> spp., <i>P. arrhenomanes</i> , <i>P. graminicola</i>
Rhizoctonia ear rot (sclerotial rot)	<i>Pythium aphanidermatum</i> = <i>P. butleri</i> L.
Rhizoctonia root rot and stalk rot	<i>Epicoccum nigrum</i>
Root rots, minor	<i>Rhizoctonia zeae</i> (teleomorph: <i>Waitea circinata</i> )
	<i>Rhizoctonia solani</i> , <i>Rhizoctonia zeae</i>
	<i>Alternaria alternata</i> , <i>Cercospora sorghi</i> , <i>Dictyochoeta fertilis</i> , <i>Fusarium acuminatum</i> (teleomorph: <i>Gibberella acuminata</i> ), <i>F. equiseti</i> (teleomorph: <i>G. intricans</i> ), <i>F. oxysporum</i> , <i>F. pallidoroseum</i> , <i>F. poae</i> , <i>F. roseum</i> , <i>G. cyanogena</i> , (anamorph: <i>F. sulphureum</i> ), <i>Microdochium bolleyi</i> , <i>Mucor</i> sp., <i>Periconia circinata</i> , <i>Phytophthora cactorum</i> , <i>P. drechsleri</i> , <i>P. nicotianae</i> var. <i>parasitica</i> , <i>Rhizopus arrhizus</i>
Rostratum leaf spot ( <i>Helminthosporium</i> leaf disease, ear and stalk rot)	<i>Setosphaeria rostrata</i> , (anamorph: <i>Exserohilum rostratum</i> = <i>Helminthosporium rostratum</i> )
Rust, common corn	<i>Puccinia sorghi</i>
Rust, southern corn	<i>Puccinia polysora</i>
Rust, tropical corn	<i>Physopella pallescens</i> , <i>P. zeae</i> = <i>Angiopsora zeae</i>
Sclerotium ear rot* (southern blight)	<i>Sclerotium rolfsii</i> Sacc. (teleomorph: <i>Athelia rolfsii</i> )
Seed rot-seedling blight	<i>Bipolaris sorokiniana</i> , <i>B. zeicola</i> = <i>Helminthosporium carbonum</i> , <i>Diplodia</i>

DISEASE	CAUSATIVE AGENT
	<i>maydis</i> , <i>Exserohilum pedicellatum</i> , <i>Exserohilum turcicum</i> = <i>Helminthosporium</i> <i>turcicum</i> , <i>Fusarium avenaceum</i> , <i>F. culmorum</i> , <i>F. moniliforme</i> , <i>Gibberella zeae</i> (anamorph: <i>F.</i> <i>graminearum</i> ), <i>Macrophomina phaseolina</i> , <i>Penicillium</i> spp., <i>Phomopsis</i> sp., <i>Pythium</i> spp., <i>Rhizoctonia solani</i> , <i>R. zeae</i> , <i>Sclerotium rolfsii</i> , <i>Spicaria</i> sp.
Selenophoma leaf spot*	<i>Selenophoma</i> sp.
Sheath rot	<i>Gaeumannomyces graminis</i>
Shuck rot	<i>Myrothecium gramineum</i>
Silage mold	<i>Monascus purpureus</i> , <i>M. ruber</i>
Smut, common	<i>Ustilago zeae</i> = <i>U. maydis</i> )
Smut, false	<i>Ustilaginoides virens</i>
Smut, head	<i>Sphacelotheca reiliana</i> = <i>Sporisorium holci-</i> <i>sorghii</i>
Southern corn leaf blight and stalk rot	<i>Cochliobolus heterostrophus</i> (anamorph: <i>Bipolaris maydis</i> = <i>Helminthosporium maydis</i> )
Southern leaf spot	<i>Stenocarpella macrospora</i> = <i>Diplodia</i> <i>macrospora</i>
Stalk rots, minor	<i>Cercospora sorghi</i> , <i>Fusarium episphaeria</i> , <i>F.</i> <i>merismoides</i> , <i>F. oxysporum</i> Schlechtend, <i>F.</i> <i>poae</i> , <i>F. roseum</i> , <i>F. solani</i> (teleomorph: <i>Nectria haematococca</i> ), <i>F. tricinctum</i> , <i>Mariannaea elegans</i> , <i>Mucor</i> sp., <i>Rhopoglyphus zeae</i> , <i>Spicaria</i> sp.
Storage rots	<i>Aspergillus</i> spp., <i>Penicillium</i> spp. and other fungi
Tar spot*	<i>Phyllachora maydis</i>
Trichoderma ear rot and root rot	<i>Trichoderma viride</i> = <i>T. lignorum</i> teleomorph: <i>Hypocrea</i> sp.
White ear rot, root and stalk rot	<i>Stenocarpella maydis</i> = <i>Diplodia zeae</i>
Yellow leaf blight	<i>Ascochyta ischaemi</i> , <i>Phyllosticta maydis</i> (teleomorph: <i>Mycosphaerella zeae-maydis</i> )
Zonate leaf spot	<i>Gloeocercospora sorghi</i>
*Not known to occur naturally on corn in the United States.	

Plant parasitic nematodes are a cause of disease in many plants, including maize. It is proposed that it would be possible to make plants resistant to these organisms through the expression of novel gene products. It is anticipated that control of nematode infestations would

be accomplished by altering the ability of the nematode to recognize or attach to a host plant and/or enabling the plant to produce nematicidal compounds, including but not limited to proteins. It is known that certain endotoxins derived from *Bacillus thuringiensis* are nematicidal (Bottjer *et al.*, 1985; U.S. Patent No. 5,831,011). Examples of nematode-associated plant diseases, for which one could introduce resistance to in a transgenic plant in accordance with the invention are given below, in Table 6.

**Table 6: Parasitic Nematodes**

DISEASE	PATHOGEN
Awl	<i>Dolichodorus</i> spp., <i>D. heterocephalus</i>
Bulb and stem (Europe)	<i>Ditylenchus dipsaci</i>
Burrowing	<i>Radopholus similis</i>
Cyst	<i>Heterodera avenae</i> , <i>H. zae</i> , <i>Punctodera chalcensis</i>
Dagger	<i>Xiphinema</i> spp., <i>X. americanum</i> , <i>X. mediterraneum</i>
False root-knot	<i>Nacobbus dorsalis</i>
Lance, Columbia	<i>Hoplolaimus columbus</i>
Lance	<i>Hoplolaimus</i> spp., <i>H. galeatus</i>
Lesion	<i>Pratylenchus</i> spp., <i>P. brachyurus</i> , <i>P. crenatus</i> , <i>P. hexincisus</i> , <i>P. neglectus</i> , <i>P. penetrans</i> , <i>P. scribneri</i> , <i>P. thornei</i> , <i>P. zae</i>
Needle	<i>Longidorus</i> spp., <i>L. breviannulatus</i>
Ring	<i>Criconemella</i> spp., <i>C. ornata</i>
Root-knot	<i>Meloidogyne</i> spp., <i>M. chitwoodi</i> , <i>M. incognita</i> , <i>M. javanica</i>
Spiral	<i>Helicotylenchus</i> spp.
Sting	<i>Belonolaimus</i> spp., <i>B. longicaudatus</i>
Stubby-root	<i>Paratrichodorus</i> spp., <i>P. christiei</i> , <i>P. minor</i> , <i>Quinisulcius acutus</i> , <i>Trichodorus</i> spp.
Stunt	<i>Tylenchorhynchus dubius</i>

#### **E. Mycotoxin Reduction/Elimination**

Production of mycotoxins, including aflatoxin and fumonisin, by fungi associated with monocotyledonous plants such as maize is a significant factor in rendering the grain not useful. These fungal organisms do not cause disease symptoms and/or interfere with the growth of the plant, but they produce chemicals (mycotoxins) that are toxic to animals. It is contemplated that inhibition of the growth of these fungi would reduce the synthesis of these toxic substances and

therefore reduce grain losses due to mycotoxin contamination. It also is proposed that it may be possible to introduce novel genes into monocotyledonous plants such as maize that would inhibit synthesis of the mycotoxin and it would be advantageous to express such genes in a tissue such as the pedicel, drive by a promoter that shows increasing activity with kernel development such as GS<sub>1-2</sub>. Further, it is contemplated that expression of a novel gene which encodes an enzyme capable of rendering the mycotoxin nontoxic would be useful in order to achieve reduced mycotoxin contamination of grain. The result of any of the above mechanisms would be a reduced presence of mycotoxins on grain.

#### **F. Grain Composition or Quality**

Genes may be introduced into monocotyledonous plants, particularly commercially important cereals such as maize, to improve the grain for which the cereal is primarily grown. A wide range of novel transgenic plants produced in this manner may be envisioned depending on the particular end use of the grain. It is especially advantageous to express such grain enhancing traits in tissues which either become or support kernels and other female reproductive tissues. In this aspect, the maize GS<sub>1-2</sub> promoter is especially useful as it has been shown to express in kernels and cob of pre-pollination maize, and in the pedicel and basal cell transfer layer of developing kernels for several days post-pollination. In general, it would be desirable to operably join the maize GS<sub>1-2</sub> promoter to genes with transport functions, for the movement or metabolism of molecules, including but not limited to, sugars, amino acids, ADP or ATP, phosphate, hormones or fatty acids.

The largest uses of maize grain are for animal feed or human food. Introduction of genes that alter the composition of the grain may greatly enhance the feed or food value. The primary components of maize grain are starch, protein, and oil. Each of these primary components of maize grain may be improved by altering its level or composition. Several examples may be mentioned for illustrative purposes, but in no way provide an exhaustive list of possibilities.

The protein of cereal grains including maize is suboptimal for feed and food purposes especially when fed to monogastric animals such as pigs, poultry, and humans. The protein is deficient in several amino acids that are essential in the diet of these species, requiring the addition of supplements to the grain. Limiting essential amino acids may include lysine, methionine, tryptophan, threonine, valine, arginine, and histidine. Some amino acids become limiting only after corn is supplemented with other inputs for feed formulations. For example, when corn is supplemented with soybean meal to meet lysine requirements methionine becomes limiting. The levels of these essential amino acids in seeds and grain may be elevated by mechanisms which include, but are not limited to, the introduction of genes to increase the biosynthesis of the amino acids, decrease the degradation of the amino acids, increase the storage of the amino acids in proteins, direct the storage of amino acids in proteins comprising a nutritionally enhanced balance of amino acids, or increase transport of the amino acids to the seeds or grain. A maize pedicel-specific promoter such as GS<sub>1-2</sub> would be especially useful for driving expression of genes affecting amino acid transport, synthesis or storage.

One mechanism for increasing the biosynthesis of the amino acids is to introduce genes that deregulate the amino acid biosynthetic pathways such that the plant can no longer adequately control the levels that are produced. This may be done by deregulating or bypassing steps in the amino acid biosynthetic pathway which are normally regulated by levels of the amino acid end product of the pathway. Examples include the introduction of genes that encode deregulated versions of the enzymes aspartokinase or dihydrodipicolinic acid (DHDP)-synthase for increasing lysine and threonine production, and anthranilate synthase for increasing tryptophan production. Reduction of the catabolism of the amino acids may be accomplished by introduction of DNA sequences that reduce or eliminate the expression of genes encoding enzymes that catalyze steps in the catabolic pathways such as the enzyme lysine-ketoglutarate reductase. It is anticipated that it may be desirable to target expression of genes relating to amino acid biosynthesis to the endosperm or embryo of the seed. More preferably, the gene will be targeted to the embryo. It will also be preferable for genes encoding proteins involved in amino acid biosynthesis to target the protein to a plastid using a plastid transit peptide sequence.



The protein composition of the grain may be altered to improve the balance of amino acids in a variety of ways including elevating expression of native proteins, decreasing expression of those with poor composition, changing the composition of native proteins, or introducing genes encoding entirely new proteins possessing superior composition. Examples may include the introduction of DNA that decreases the expression of members of the zein family of storage proteins. This DNA may encode ribozymes or antisense sequences directed to impairing expression of zein proteins or expression of regulators of zein expression such as the opaque-2 gene product. It also is proposed that the protein composition of the grain may be modified through the phenomenon of co-suppression, *i.e.*, inhibition of expression of an endogenous gene through the expression of an identical structural gene or gene fragment introduced through transformation (Goring *et al.*, 1991; PCT Publication WO 98/26064). Additionally, the introduced DNA may encode enzymes which degrade zeins. The decreases in zein expression that are achieved may be accompanied by increases in proteins with more desirable amino acid composition or increases in other major seed constituents such as starch. Alternatively, a chimeric gene may be introduced that comprises a coding sequence for a native protein of adequate amino acid composition such as for one of the globulin proteins or 10 kD delta zein or 20 kD delta zein or 27 kD gamma zein of maize and a promoter or other regulatory sequence designed to elevate expression of said protein. The coding sequence of the gene may include additional or replacement codons for essential amino acids. Further, a coding sequence obtained from another species, or, a partially or completely synthetic sequence encoding a completely unique peptide sequence designed to enhance the amino acid composition of the seed may be employed. It is anticipated that it may be preferable to target expression of these transgenes encoding proteins with superior composition to the endosperm of the seed.

The introduction of genes that alter the oil content of the grain may be of value. Increases in oil content may result in increases in metabolizable-energy-content and density of the seeds for use in feed and food. The introduced genes may encode enzymes that remove or reduce rate-limitations or regulated steps in fatty acid or lipid biosynthesis. Such genes may include, but are not limited to, those that encode acetyl-CoA carboxylase, ACP-acyltransferase,  $\beta$ -ketoacyl-ACP synthase, plus other well known fatty acid biosynthetic activities. Other possibilities are genes

that encode proteins that do not possess enzymatic activity such as acyl carrier protein. Genes may be introduced that alter the balance of fatty acids present in the oil providing a more healthful or nutritive feedstuff. The introduced DNA also may encode sequences that block expression of enzymes involved in fatty acid biosynthesis, altering the proportions of fatty acids present in the grain such as described below. Some other examples of genes specifically contemplated by the inventors for use in creating transgenic plants with altered oil composition traits include 2-acetyltransferase, oleosin, pyruvate dehydrogenase complex, acetyl CoA synthetase, ATP citrate lyase, ADP-glucose pyrophosphorylase and genes of the carnitine-CoA-acetyl-CoA shuttles. It is anticipated that expression of genes related to oil biosynthesis will be targeted to the plastid, using a plastid transit peptide sequence and preferably expressed in the seed embryo. A pedicel-specific promoter such as maize GS<sub>1-2</sub> may be beneficial for use in increasing the transport of oil from the parent plant into the developing kernel for storage or use.

Genes may be introduced that enhance the nutritive value of the starch component of the grain, for example by increasing the degree of branching, resulting in improved utilization of the starch, for example, in cows by delaying its metabolism. It is contemplated that alteration of starch structure may improve the wet milling properties of grain or may produce a starch composition with improved qualities for industrial utilization. It is anticipated that expression of genes related to starch biosynthesis will preferably be targeted to the endosperm of the seed.

Besides affecting the major constituents of the grain, genes may be introduced that affect a variety of other nutritive, processing, or other quality aspects of the grain as used for feed or food. For example, pigmentation of the grain may be increased or decreased. Enhancement and stability of yellow pigmentation is desirable in some animal feeds and may be achieved by introduction of genes that result in enhanced production of xanthophylls and carotenes by eliminating rate-limiting steps in their production. Such genes may encode altered forms of the enzymes phytoene synthase, phytoene desaturase, or lycopene synthase. Alternatively, unpigmented white corn is desirable for production of many food products and may be produced by the introduction of DNA which blocks or eliminates steps in pigment production pathways.

Most of the phosphorous content of the grain is in the form of phytate, a form of phosphate storage that is not metabolized by monogastric animals. Therefore, in order to increase the availability of seed phosphate, it is anticipated that one will desire to decrease the amount of phytate in seed and increase the amount of free phosphorous. It is anticipated that one can decrease the expression or activity of one of the enzymes involved in the synthesis of phytate. For example, suppression of expression of the gene encoding inositol phosphate synthetase (INOPS) may lead to an overall reduction in phytate accumulation. It is anticipated that antisense or sense suppression of gene expression may be used. Alternatively, one may express a gene in corn seed which will be activated, *e.g.*, by pH, in the gastric system of a monogastric animal and will release phosphate from phytate, *e.g.*, phytase. It is further contemplated that one may provide an alternate storage form for phosphate in the grain, wherein the storage form is more readily utilized by a monogastric animal.

Feed or food comprising primarily maize or other cereal grains possesses insufficient quantities of vitamins and must be supplemented to provide adequate nutritive value. Introduction of genes that enhance vitamin biosynthesis in seeds may be envisioned including, for example, vitamins A, E, B<sub>12</sub>, choline, and the like. Maize grain also does not possess sufficient mineral content for optimal nutritive value. Genes that affect the accumulation or availability of compounds containing phosphorus, sulfur, calcium, manganese, zinc, and iron among others would be valuable. An example may be the introduction of a gene that reduced phytic acid production or encoded the enzyme phytase which enhances phytic acid breakdown. These genes would increase levels of available phosphate in the diet, reducing the need for supplementation with mineral phosphate.

Numerous other examples of improvement of maize or other cereals for feed and food purposes might be described. The improvements may not even necessarily involve the grain, but may, for example, improve the value of the corn for silage. Introduction of DNA to accomplish this might include sequences that alter lignin production such as those that result in the "brown midrib" phenotype associated with superior feed value for cattle. As the GS<sub>1-2</sub> promoter appears to show activity in the cob of both pre- and post-pollinated corn, it may provide a mechanism to

drive expression of genes to increase the nutritional value of the cob for use as silage or other purposes.

In addition to direct improvements in feed or food value, genes also may be introduced which improve the processing of corn and improve the value of the products resulting from the processing. The primary method of processing corn is via wetmilling. Maize may be improved though the expression of novel genes that increase the efficiency and reduce the cost of processing such as by decreasing steeping time.

Improving the value of wetmilling products may include altering the quantity or quality of starch, oil, corn gluten meal, or the components of corn gluten feed. Elevation of starch may be achieved through the identification and elimination of rate limiting steps in starch biosynthesis or by decreasing levels of the other components of the grain resulting in proportional increases in starch. An example of the former may be the introduction of genes encoding ADP-glucose pyrophosphorylase enzymes with altered regulatory activity or which are expressed at higher level. Examples of the latter may include selective inhibitors of, for example, protein or oil biosynthesis expressed during later stages of kernel development.

The properties of starch may be beneficially altered by changing the ratio of amylose to amylopectin, the size of the starch molecules, or their branching pattern. Through these changes a broad range of properties may be modified which include, but are not limited to, changes in gelatinization temperature, heat of gelatinization, clarity of films and pastes, rheological properties, and the like. To accomplish these changes in properties, genes that encode granule-bound or soluble starch synthase activity or branching enzyme activity may be introduced alone or combination. DNA such as antisense constructs also may be used to decrease levels of endogenous activity of these enzymes. The introduced genes or constructs may possess regulatory sequences that time their expression to specific intervals in starch biosynthesis and starch granule development. Furthermore, it may be worthwhile to introduce and express genes that result in the *in vivo* derivatization, or other modification, of the glucose moieties of the starch molecule. The covalent attachment of any molecule may be envisioned, limited only by the

existence of enzymes that catalyze the derivatizations and the accessibility of appropriate substrates in the starch granule. Examples of important derivations may include the addition of functional groups such as amines, carboxyls, or phosphate groups which provide sites for subsequent *in vitro* derivatizations or affect starch properties through the introduction of ionic charges. Examples of other modifications may include direct changes of the glucose units such as loss of hydroxyl groups or their oxidation to aldehyde or carboxyl groups.

Oil is another product of wetmilling of corn, the value of which may be improved by introduction and expression of genes. The quantity of oil that can be extracted by wetmilling may be elevated by approaches as described for feed and food above. Oil properties also may be altered to improve its performance in the production and use of cooking oil, shortenings, lubricants or other oil-derived products or improvement of its health attributes when used in the food-related applications. Novel fatty acids also may be synthesized which upon extraction can serve as starting materials for chemical syntheses. The changes in oil properties may be achieved by altering the type, level, or lipid arrangement of the fatty acids present in the oil. This in turn may be accomplished by the addition of genes that encode enzymes that catalyze the synthesis of novel fatty acids and the lipids possessing them or by increasing levels of native fatty acids while possibly reducing levels of precursors. Alternatively, DNA sequences may be introduced which slow or block steps in fatty acid biosynthesis resulting in the increase in precursor fatty acid intermediates. Genes that might be added include desaturases, epoxidases, hydratases, dehydratases, and other enzymes that catalyze reactions involving fatty acid intermediates. Representative examples of catalytic steps that might be blocked include the desaturations from stearic to oleic acid and oleic to linolenic acid resulting in the respective accumulations of stearic and oleic acids. Another example is the blockage of elongation steps resulting in the accumulation of C<sub>8</sub> to C<sub>12</sub> saturated fatty acids.

Improvements in the other major corn wetmilling products, corn gluten meal and corn gluten feed, also may be achieved by the introduction of genes to obtain novel corn plants. Representative possibilities include but are not limited to those described above for improvement of food and feed value.

In addition, it may further be considered that the corn plant be used for the production or manufacturing of useful biological compounds that were either not produced at all, or not produced at the same level, in the corn plant previously. The novel corn plants producing these compounds are made possible by the introduction and expression of genes by corn transformation methods. The vast array of possibilities include but are not limited to any biological compound which is presently produced by any organism such as proteins, nucleic acids, primary and intermediary metabolites, carbohydrate polymers, *etc.* The compounds may be produced by the plant, extracted upon harvest and/or processing, and used for any presently recognized useful purpose such as pharmaceuticals, fragrances, and industrial enzymes to name a few. In some cases, it may be beneficial to limit the production, manufacture or storage of useful biological compounds to a certain tissue; the maize GS<sub>1,2</sub> promoter will be useful to allow expression of operably linked genes in the female reproductive tissues and especially the developing kernel and cob.

Further possibilities to exemplify the range of grain traits or properties potentially encoded by introduced genes in transgenic plants include grain with less breakage susceptibility for export purposes or larger grit size when processed by dry milling through introduction of genes that enhance  $\gamma$ -zein synthesis, popcorn with improved popping quality and expansion volume through genes that increase pericarp thickness, corn with whiter grain for food uses through introduction of genes that effectively block expression of enzymes involved in pigment production pathways, and improved quality of alcoholic beverages or sweet corn through introduction of genes which affect flavor such as the shrunken 1 gene (encoding sucrose synthase) or shrunken 2 gene (encoding ADPG pyrophosphorylase) for sweet corn.

#### **G. Plant Agronomic Characteristics**

Two of the factors determining where crop plants can be grown are the average daily temperature during the growing season and the length of time between frosts. Within the areas where it is possible to grow a particular crop, there are varying limitations on the maximal time the crop has available to grow to maturity and be harvested. For example, maize to be grown in a

particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, corn of varying maturities is developed for different growing locations. Apart from the need to dry down sufficiently to permit harvest, it is desirable to have maximal drying take place in the field to minimize the amount of energy required for additional post-harvest drying. Also, the more readily the grain can dry down, the more time there is available for growth and seed maturation. It is considered that genes that influence maturity and/or dry down can be identified and introduced into corn or other plants using transformation techniques to create new varieties adapted to different growing locations or the same growing location, but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially useful, *e.g.*, the liguleless and rough sheath genes that have been identified in corn.

It is contemplated that genes may be introduced into plants that would improve standability and other plant growth characteristics. Expression of novel genes in maize which confer stronger stalks, improved root systems, or prevent or reduce ear droppage would be of great value to the farmer. It is proposed that introduction and expression of genes that increase the total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In addition, the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further increase gains in productivity. It is contemplated that expression of a phytochrome gene in corn may be advantageous. Expression of such a gene may reduce apical dominance, confer semidwarfism on a plant, and increase shade tolerance (U.S. Patent No. 5,268,526). Such approaches would allow for increased plant populations in the field.

Delay of late season vegetative senescence would increase the flow of assimilate into the grain and thus increase yield. It is proposed that overexpression of genes within corn that are associated with "stay green" or the expression of any gene that delays senescence would be advantageous. For example, a nonyellowing mutant has been identified in *Festuca pratensis* (Davies *et al.*, 1990). Expression of this gene as well as others may prevent premature breakdown of chlorophyll and thus maintain canopy function.

## **H. Nutrient Utilization**

5 The ability to utilize available nutrients may be a limiting factor in growth of monocotyledonous plants such as maize. It is proposed that it would be possible to alter nutrient uptake, tolerate pH extremes, mobilization through the plant, storage pools, and availability for metabolic activities by the introduction of novel genes. These modifications would allow a plant such as maize to more efficiently utilize available nutrients. It is contemplated that an increase in  
10 the activity of, for example, an enzyme that is normally present in the plant and involved in nutrient utilization would increase the availability of a nutrient. An example of such an enzyme would be phytase. It further is contemplated that enhanced nitrogen utilization by a plant is desirable. Expression of a glutamate dehydrogenase gene in corn, *e.g.*, *E. coli gdhA* genes, may lead to increased fixation of nitrogen in organic compounds. Furthermore, expression of *gdhA* in  
15 corn may lead to enhanced resistance to the herbicide glufosinate by incorporation of excess ammonia into glutamate, thereby detoxifying the ammonia. It also is contemplated that expression of a novel gene may make a nutrient source available that was previously not accessible, *e.g.*, an enzyme that releases a component of nutrient value from a more complex molecule, perhaps a macromolecule. It would be beneficial to utilize a developmentally regulated, female tissue specific promoter such as the GS<sub>1-2</sub> promoter to express nutrient utilization genes as  
20 described in but not limited to this discussion, in a manner in which expression may enhance kernel development or other features of the developing, mature or germinating kernel..

## **I. Female Sterility**

25 It is known in the art that male sterility is useful in the production of hybrid seed. However, female sterility may also be useful in the production of hybrid seed as well. Typically, hybrid production fields are planted such that male parent plants and female parent plants are in alternating blocks of rows in the field. Usually, the parent plants are of different inbred lines although they may consist of other desired lines as well. The female parent plants are male sterile  
30 and ideally, do not participate in the pollination of the ears on any plants; the pollen from the male



parent plant is the desired pollen for fertilization of the female parent plants. The fertilized ears and thus the seed, collected from the female plant is hybrid.

In addition to the production of the desired hybrid corn on the female parent plant, the pollen from the male parent plant also fertilizes the ear of the male parent plant. The ear and seed which develop on the male plant is thus the result of a self pollination and is not hybrid as the female did not donate pollen. During harvest, it is necessary to collect only the ears and seed from the hybrid plants and to not collect the ears and seed from the self-fertilized plants.

It is anticipated that the use of a female sterile male parent plant would be useful in the production of hybrid corn. If the male parent plants did not produce female reproductive tissues, self-pollination would not occur and harvesting could be simplified. A female tissue specific promoter, such as the pedicel and developing kernel specific GS<sub>1-2</sub> promoter of this invention, may be used to drive expression of genes which could interrupt the development of female reproductive tissues such as the cob or kernel.

#### **J. Negative Selectable Markers**

Introduction of genes encoding traits that can be selected against may be useful for eliminating undesirable linked genes. It is contemplated that when two or more genes are introduced together by cotransformation that the genes will be linked together on the host chromosome. For example, a gene encoding *Bt* that confers insect resistance on the plant may be introduced into a plant together with a *bar* gene that is useful as a selectable marker and confers resistance to the herbicide Liberty® on the plant. However, it may not be desirable to have an insect resistant plant that also is resistant to the herbicide Liberty®. It is proposed that one also could introduce an antisense *bar* coding region that is expressed in those tissues where one does not want expression of the *bar* gene product, *e.g.*, in whole plant parts. Hence, although the *bar* gene is expressed and is useful as a selectable marker, it is not useful to confer herbicide resistance on the whole plant. The *bar* antisense gene is a negative selectable marker.

It also is contemplated that negative selection is necessary in order to screen a population of transformants for rare homologous recombinants generated through gene targeting. For example, a homologous recombinant may be identified through the inactivation of a gene that was previously expressed in that cell. The antisense construct for neomycin phosphotransferase II (NPT II) has been investigated as a negative selectable marker in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Xiang, and Guerra, 1993). In this example, both sense and antisense NPT II genes are introduced into a plant through transformation and the resultant plants are sensitive to the antibiotic kanamycin. An introduced gene that integrates into the host cell chromosome at the site of the antisense NPT II gene, and inactivates the antisense gene, will make the plant resistant to kanamycin and other aminoglycoside antibiotics. Therefore, rare, site-specific recombinants may be identified by screening for antibiotic resistance. Similarly, any gene, native to the plant or introduced through transformation, that when inactivated confers resistance to a compound, may be useful as a negative selectable marker.

It is contemplated that negative selectable markers also may be useful in other ways. One application is to construct transgenic lines in which one could select for transposition to unlinked sites. In the process of tagging it is most common for the transposable element to move to a genetically linked site on the same chromosome. A selectable marker for recovery of rare plants in which transposition has occurred to an unlinked locus would be useful. For example, the enzyme cytosine deaminase may be useful for this purpose. In the presence of this enzyme the non-phytotoxic compound 5-fluorocytosine is converted to 5-fluorouracil which is toxic to plant and animal cells. If a transposable element is linked to the gene for the enzyme cytosine deaminase, one may select for transposition to unlinked sites by selecting for transposition events in which the resultant plant is now resistant to 5-fluorocytosine. The parental plants and plants containing transpositions to linked sites will remain sensitive to 5-fluorocytosine. Resistance to 5-fluorocytosine is due to loss of the cytosine deaminase gene through genetic segregation of the transposable element and the cytosine deaminase gene. Other genes that encode proteins that render the plant sensitive to a certain compound will also be useful in this context. For example, T-DNA gene 2 from *Agrobacterium tumefaciens* encodes a protein that catalyzes the conversion

of  $\alpha$ -naphthalene acetamide (NAM) to  $\alpha$ -naphthalene acetic acid (NAA) renders plant cells sensitive to high concentrations of NAM (Depicker *et al.*, 1988).

It also is contemplated that negative selectable markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such as Ac, Master Mu, or En/Spn with a negative selectable marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. It is proposed that this would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as Ds, but stable integration of the autonomous element is not desired. The presence of the autonomous element may not be desired in order to stabilize the defective element, *i.e.*, prevent it from further transposing. However, it is proposed that if stable integration of an autonomous transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.

#### **K. Non-Protein-Expressing Sequences**

DNA may be introduced into plants for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes. However, as detailed below, DNA need not be expressed to effect the phenotype of a plant.

##### **1. Antisense RNA**

Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant.

Reduction of the enzyme activity may reduce or eliminate products of the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited above are provided only by way of example and do not represent the full range of applications.

## 2. Ribozymes

Genes also may be constructed or isolated, which when transcribed, produce RNA enzymes (ribozymes) which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected messenger RNAs can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including, but not limited to, the polypeptides cited above.

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes.

Several different ribozyme motifs have been described with RNA cleavage activity (Symons, 1992). Examples include sequences from the Group I self splicing introns including Tobacco Ringspot Virus (Prody *et al.*, 1986), Avocado Sunblotch Viroid (Palukaitis *et al.*, 1979; Symons, 1981), and Lucerne Transient Streak Virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozyme based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992, Yuan and Altman, 1994, U.S. Patents 5,168,053 and 5,624,824), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and Hepatitis Delta virus based ribozymes (U.S. Patent No. 5,625,047). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira *et al.*, 1994; Thompson *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complimentary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozyme, the cleavage site is a dinucleotide sequence on the target RNA is a uracil (U) followed by either an adenine, cytosine or uracil (A,C or U) (Perriman *et al.*, 1992; Thompson *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible.

Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in

down regulating a given gene is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

### 3. Induction of Gene Silencing

It also is possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by the mechanism of co-suppression. It has been demonstrated in tobacco, tomato, petunia, and corn (Goring *et al.*, 1991; Smith *et al.*, 1990; Napoli *et al.*, 1990; van der Krol *et al.*, 1990; PCT Publication WO 98/26064) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

### 4. Non-RNA-Expressing Sequences

DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be inserted into a gene to cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, *e.g.*, using the introduced DNA sequence as a PCR primer target sequence together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta *et al.*, 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired, may be isolated, cloned and manipulated as desired. The utility of DNA elements introduced into an organism for purposes of gene tagging is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, *i.e.*, transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

It is contemplated that unexpressed DNA sequences, including novel synthetic sequences, could be introduced into cells as proprietary "labels" of those cells and plants and seeds thereof.

It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that labeled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief *et al.*, 1989), which can be positioned around an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependent effects upon incorporation into the plant genome (Stief *et al.*, 1989; Phi-Van *et al.*, 1990).

#### **IV. Assays of Transgene Expression**

Assays may be employed with the instant invention for determination of the relative efficiency of transgene expression. For example, assays may be used to determine the efficacy of deletion mutants of the GS<sub>1-2</sub> promoter in directing expression of exogenous proteins. Similarly, one could produce random or site-specific mutants of the GS<sub>1-2</sub> promoter of the invention and assay the efficacy of the mutants in the expression of a given transgene. Alternatively, assays could be used to determine the efficacy of the GS<sub>1-2</sub> promoter in directing protein expression when used in conjunction with various different enhancers, 3' untranslated regions or other types of elements potentially used in the preparation of transformation constructs.

For plants, expression assays may comprise a system utilizing embryogenic or non-embryogenic cells, or alternatively, whole plants. An advantage of using cellular assays is that regeneration of large numbers of plants is not required. However, the systems are limited in that promoter activity in the non-regenerated cells may not directly correlate with expression in a plant. Additionally, assays of tissue or developmental specific promoters are generally not feasible.

The biological sample to be assayed may comprise nucleic acids isolated from the cells of any plant material according to standard methodologies (Sambrook and Russell, 2001; Ausubel *et al.*, 2001). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment of the invention, the RNA is whole cell RNA; in another, it is poly-A RNA. Normally, the nucleic acid is amplified.

Depending on the format, the specific nucleic acid of interest is identified in the sample directly using amplification or with a second, known nucleic acid following amplification. Next, the identified product is detected. In certain applications, the detection may be performed by visual means (*e.g.*, ethidium bromide staining of a gel). Alternatively, the detection may involve indirect identification of the product *via* chemiluminescence, radioactive scintigraphy of radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (Affymax Technology; Bellus, 1994).

Following detection, one may compare the results seen in a given plant with a statistically significant reference group of non-transformed control plants. Typically, the non-transformed control plants will be of a genetic background similar to the transformed plants. In this way, it is possible to detect differences in the amount or kind of protein detected in various transformed plants. Alternatively, clonal cultures of cells, for example, callus or an immature embryo, may be compared to other cells samples.

As indicated, a variety of different assays are contemplated in the screening of cells or plants of the current invention and associated promoters. These techniques may in cases be used to detect for both the presence and expression of the particular genes as well as rearrangements that may have occurred in the gene construct. The techniques include but are not limited to, fluorescent *in situ* hybridization (FISH), direct DNA sequencing, pulsed field gel electrophoresis (PFGE) analysis, Southern or Northern blotting, single-stranded conformation analysis (SSCA),



RNase protection assay, allele-specific oligonucleotide (ASO), dot blot analysis, denaturing gradient gel electrophoresis, RFLP and PCR-SSCP.

#### **A. Quantitation of Gene Expression with Relative Quantitative RT- PCR**

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific mRNA species isolated from plants. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed. In this way, a promoters expression profile can be rapidly identified, as can the efficacy with which the promoter directs transgene expression.

In PCR, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the

concentration of the PCR products and the relative mRNA abundances is only true in the linear range of the PCR reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met before the relative abundances of a mRNA species can be determined by RT-PCR for a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR study to successfully determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR study is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample.

Most protocols for competitive PCR utilize internal PCR standards that are approximately as abundant as the target. These strategies are effective if the products of the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR assay for plant tissue. The problems inherent in plant tissue samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-

amplification of a reliable internal control, preferably of larger size than the target). Both of these problems are overcome if the RT-PCR is performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR assay with an external standard protocol. These assays sample the PCR products in the linear portion of their amplification curves. The number of PCR cycles that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR assays can be superior to those derived from the relative quantitative RT-PCR assay with an internal standard.

One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

### **B. Marker Gene Expression**

Marker genes represent an efficient means for assaying the expression of transgenes. Using, for example, a selectable marker gene, one could quantitatively determine the resistance conferred upon a plant or plant cell by a construct comprising the selectable marker coding region operably linked to the promoter to be assayed, *e.g.*, a GS<sub>1-2</sub> promoter. Alternatively, various plant

parts could be exposed to a selective agent and the relative resistance provided in these parts quantified, thereby providing an estimate of the tissue specific expression of the promoter.

Screenable markers constitute another efficient means for quantifying the expression of a given transgene. Potentially any screenable marker could be expressed and the marker gene product quantified, thereby providing an estimate of the efficiency with which the promoter directs expression of the transgene. Quantification can readily be carried out using either visual means, or, for example, a photon counting device.

A preferred screenable marker gene assay for use with the current invention constitutes the use of the screenable marker gene  $\beta$ -glucuronidase (Jefferson *et al.*, 1986; *uidA* gene; the product of which is commonly referred to as GUS). Detection of GUS activity can be performed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as the substrate for the GUS enzyme, yielding a blue precipitate inside of cells containing GUS activity. This assay has been described in detail (Jefferson, 1987). The blue coloration can then be visually scored, and estimates of expression efficiency thereby provided. GUS activity also can be determined by immunoblot analysis or a fluorometric GUS specific activity assay (Jefferson, 1987).

### **C. Purification and Assays of Proteins**

One means for determining the efficiency with which a particular transgene is expressed is to purify and quantify a polypeptide expressed by the transgene. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; and isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide being assayed always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small

fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from

castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyl binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

5

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

10

25094423.1

## **V. Methods for Plant Transformation**

Suitable methods for plant transformation for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaepler *et al.*, 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, maize cells as well as those of virtually any other plant species may be stably transformed, and these cells developed into transgenic plants. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

### **A. Electroporation**

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Patent No. 5,384,253, incorporated herein by reference in its entirety) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation by mechanical wounding.

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or



mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, 1995; D'Halluin *et al.*, 1992), wheat (Zhou *et al.*, 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.*, 1987) and tobacco (Lee *et al.*, 1989).

5

One also may employ protoplasts for electroporation transformation of plants (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in PCT Publication WO 92/17598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazzeri, 1995), sorghum (Battraw and Hall, 1991), maize (Bhattacharjee *et al.*, 1997), wheat (He *et al.*, 1994) and tomato (Tsukada, 1989).

10

#### **B. Microprojectile Bombardment**

A preferred method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Publication WO 95/06128; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

15

20

25

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

30

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics Particle Delivery System (BioRad, Hercules, CA), which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with monocot plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing the damage inflicted on the recipient cells by projectiles that are too large.

For microprojectile bombardment, one will attach (*i.e.*, “coat”) DNA to the microprojectiles such that it is delivered to recipient cells in a form suitable for transformation thereof. In this respect, at least some of the transforming DNA must be available to the target cell for transformation to occur, while at the same time during delivery the DNA must be attached to the microprojectile. Therefore, availability of the transforming DNA from the microprojectile may comprise the physical reversal of bonds between transforming DNA and the microprojectile following delivery of the microprojectile to the target cell. This need not be the case, however, as availability to a target cell may occur as a result of breakage of unbound segments of DNA or of other molecules which comprise the physical attachment to the microprojectile. Availability may further occur as a result of breakage of bonds between the transforming DNA and other molecules, which are either directly or indirectly attached to the microprojectile. It further is contemplated that transformation of a target cell may occur by way of direct recombination between the transforming DNA and the genomic DNA of the recipient cell. Therefore, as used herein, a “coated” microprojectile will be one which is capable of being used to transform a target cell, in that the transforming DNA will be delivered to the target cell, yet will be accessible to the target cell such that transformation may occur.

Any technique for coating microprojectiles which allows for delivery of transforming DNA to the target cells may be used. Methods for coating microprojectiles which have been

demonstrated to work well with the current invention have been specifically disclosed herein. DNA may be bound to microprojectile particles using alternative techniques, however. For example, particles may be coated with streptavidin and DNA end labeled with long chain thiol cleavable biotinylated nucleotide chains. The DNA adheres to the particles due to the streptavidin-biotin interaction, but is released in the cell by reduction of the thiol linkage through reducing agents present in the cell.

Alternatively, particles may be prepared by functionalizing the surface of a gold oxide particle, providing free amine groups. DNA, having a strong negative charge, binds to the functionalized particles. Furthermore, charged particles may be deposited in controlled arrays on the surface of mylar flyer disks used in the PDS-1000 Biolistics device, thereby facilitating controlled distribution of particles delivered to target tissue.

As disclosed above, it further is proposed, that the concentration of DNA used to coat microprojectiles may influence the recovery of transformants containing a single copy of the transgene. For example, a lower concentration of DNA may not necessarily change the efficiency of the transformation, but may instead increase the proportion of single copy insertion events. In this regard, approximately 1 ng to 2000 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles. In other embodiments of the invention, approximately 2.5 ng to 1000 ng, 2.5 ng to 750 ng, 2.5 ng to 500 ng, 2.5 ng to 250 ng, 2.5 ng to 100 ng, or 2.5 ng to 50 ng of transforming DNA may be used per each 1.8 mg of starting microprojectiles.

Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Publication WO 95/06128), barley (Ritala *et al.*, 1994; Hensgens *et al.*, 1993), wheat (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety), rice (Hensgens *et al.*, 1993), oat (Torbet *et al.*, 1995; Torbet *et al.*, 1998), rye (Hensgens *et al.*, 1993), sugarcane (Bower *et al.*, 1992), and sorghum (Casa *et al.*, 1993; Hagio *et al.*, 1991); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Buising and Benbow, 1994), soybean (U.S. Patent No.

5,322,783, specifically incorporated herein by reference in its entirety), sunflower (Knittel *et al.* 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (Van Eck *et al.* 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

5

For microprojectile bombardment transformation in accordance with the current invention, both physical and biological parameters may be optimized. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, such as the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, the orientation of an immature embryo or other target tissue relative to the particle trajectory, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

10

15

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as DNA concentration, gap distance, flight distance, tissue distance, and helium pressure. It further is contemplated that the grade of helium may effect transformation efficiency. One also may optimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation.

20

25

### **C. *Agrobacterium*-mediated Transformation**

*Agrobacterium*-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for

30

25094423.1

example, the methods described by Fraley *et al.*, (1985), Rogers *et al.*, (1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

*Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.*, 1997; Zhang *et al.*, 1997; U.S. Patent No. 5,591,616, specifically incorporated herein by reference in its entirety), wheat (McCormac *et al.*, 1998), barley (Tingay *et al.*, 1997; McCormac *et al.*, 1998), and maize (Ishida *et al.*, 1996).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

#### **D. Other Transformation Methods**

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these

treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts have been described (Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 1993 and U.S. Patent No. 5,508,184; each specifically incorporated herein by reference in its entirety). Examples of the use of direct uptake transformation of cereal protoplasts include transformation of rice (Ghosh-Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppeler, 1990; Kaeppeler *et al.*, 1992; U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cell are punctured. This technique has been used successfully with, for example, the monocot cereals maize (PCT Publication WO 95/06128, specifically incorporated herein by reference in its entirety; Thompson, 1995) and rice (Nagatani, 1997).

## **VI. Recipient Cells for Transformation**

Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium usually is a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth,

and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, plant cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures typically requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH, but also by whether media is solid or liquid. Table 7 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

Recipient cell targets include, but are not limited to, meristem cells, Type I, Type II, and Type III callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. It is contemplated that any cell from which a fertile plant may be regenerated is useful as a recipient cell. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the like. Those cells which are capable of proliferating as callus also are recipient cells for genetic transformation. The present invention provides techniques for transforming immature embryos and subsequent regeneration of fertile transgenic plants. Transformation of immature embryos obviates the need for long term development of recipient cell cultures. Pollen, as well as its precursor cells, microspores, may be capable of functioning as recipient cells for genetic transformation, or as vectors to carry foreign DNA for incorporation during fertilization. Direct pollen transformation would obviate the need for cell culture. Meristematic cells (*i.e.*, plant cells capable of continual cell division and characterized by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, *etc.*) may represent

another type of recipient plant cell. Because of their undifferentiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an *in vitro* meristematic cell system, retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

Cultured plant cells that can serve as recipient cells for transforming with desired DNA segments may be any plant cells including corn cells, and more specifically, cells from *Zea mays* L. Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. An example of non-embryogenic cells are certain Black Mexican Sweet (BMS) corn cells.

The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, *e.g.*, as recipient cells for transformation, has been described in U.S. Patent No. 5,134,074; and U.S. Patent No. 5,489,520; each of which is incorporated herein by reference in its entirety.

Certain techniques may be used that enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of recipient cells for use in, microprojectile transformation. Suspension culturing, particularly using the media disclosed herein, may improve the ratio of recipient to non-recipient cells in any given population. Manual selection techniques which can be employed to select recipient cells may include, *e.g.*, assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation also is a possible method of selecting for recipient cells.

Manual selection of recipient cells, *e.g.*, by selecting embryogenic cells from the surface of a Type II callus, is one means that may be used in an attempt to enrich for recipient cells prior to culturing (whether cultured on solid media or in suspension). The preferred cells may be those



located at the surface of a cell cluster, and may further be identifiable by their lack of differentiation, their size and dense cytoplasm. The preferred cells will generally be those cells which are less differentiated, or not yet committed to differentiation. Thus, one may wish to identify and select those cells which are cytoplasmically dense, relatively unvacuolated with a high nucleus to cytoplasm ratio (*e.g.*, determined by cytological observations), small in size (*e.g.*, 10-20  $\mu$ m), and capable of sustained divisions and somatic proembryo formation.

It is proposed that other means for identifying such cells also may be employed. For example, through the use of dyes, such as Evan's blue, which are excluded by cells with relatively non-permeable membranes, such as embryogenic cells, and taken up by relatively differentiated cells such as root-like cells and snake cells (so-called due to their snake-like appearance).

Other possible means of identifying recipient cells include the use of isozyme markers of embryogenic cells, such as glutamate dehydrogenase, which can be detected by cytochemical stains (Fransz *et al.*, 1989). However, it is cautioned that the use of isozyme markers including glutamate dehydrogenase may lead to some degree of false positives from non-embryogenic cells such as rooty cells which nonetheless have a relatively high metabolic activity.

#### **A. Culturing Cells to be Recipients for Transformation**

The ability to prepare and cryopreserve cultures of plant cells is important to certain aspects of the present invention, in that it provides a means for reproducibly and successfully preparing cells for transformation. A variety of different types of media have been previously developed and may be employed in carrying out various aspects of the invention. The following table, Table 7, sets forth the composition of the media preferred by the inventor for carrying out these aspects of the invention.

**Table 7: Tissue Culture Media**

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
127	MS	--	5.8	MS salts 0.65 mg/L niacin, 0.125 mg/L pyridoxine-HCl 0.125 mg/L thiamine-HCl 0.125 mg/L Ca pantothenate 150 mg L-asparagine 100 mg myo-inositol 10 g glucose 20 g L-maltose 6 g PHYTAGAR™
211	N6	2%	5.8	1 mg 2,4-D 0.5 mg niacin 1.0 mg thiamine 0.91 g L-asparagine 100 mg myo-inositol 0.5 g MES 100 mg/L casein hydrolysate 1.6 g MgCl <sub>2</sub> - 6H <sub>2</sub> O 0.69 g L-proline 2g Gelgro

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
217	N6	2%	5.8	N6 salts 1 mg/L thiamine-HCl 0.5 mg/L nicotinic acid 3.52 mg/L benzylaminopurine, 0.91 g/L L-asparagine monohydrate 100 mg/L myo-inositol 0.5 g/L MES 1.6 g/L MgCl <sub>2</sub> ·6H <sub>2</sub> O 100 mg/L casein hydrolysate 0.69 g/L L-proline 20 g/L sucrose 2 g/L GELGRO™
(media#)L	--	--	--	Media supplemented with 500 mg/L paromomycin
(media#)S	--	12%	--	Media supplemented with 12% sucrose
(media#)T	--	--	--	Media supplemented with 100 mg/L paromomycin
(media#)V	--	--	--	Media supplemented with 16.9 mg/L silver nitrate

\*Basic MS medium described in Murashige and Skoog (1962). This medium is typically modified by decreasing the NH<sub>4</sub>NO<sub>3</sub> from 1.64 g/l to 1.55 g/l, and omitting the pyridoxine HCl, nicotinic acid, myo-inositol and glycine.

\*\* NAA = Naphthol Acetic Acid  
2,4-D = 2, 4-Dichlorophenoxyacetic Acid  
MES = 2-(4-morpholino)-ethane sulfonic acid

\*\*\* Supplements are assigned a letter code; combination of a media with a supplement appends the supplement letter to the media number. Thus, media #211 containing 16.9 mg/L silver nitrate (code V) is abbreviated #211V.

A number of exemplary maize cultures which may be used for transformation have been developed and are disclosed in PCT publication WO 95/06128, the disclosure of which is specifically incorporated herein by reference.

## **B. Media**

In certain embodiments of the current invention, recipient cells may be selected following growth in culture. Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components (see Table 7), but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.* (1975) and MS media (Murashige and Skoog, 1962). It has been discovered that media such as MS which have a high ammonia/nitrate ratio are counterproductive to the generation of recipient cells in that they promote loss of morphogenic capacity. N6 media, on the other hand, has a somewhat lower ammonia/nitrate ratio, and is contemplated to promote the generation of recipient cells by maintaining cells in a proembryonic state capable of sustained divisions.

## **C. Maintenance**

The method of maintenance of cell cultures may contribute to their utility as sources of recipient cells for transformation. Manual selection of cells for transfer to fresh culture medium, frequency of transfer to fresh culture medium, composition of culture medium, and environmental factors including, but not limited to, light quality and quantity and temperature are all important factors in maintaining callus and/or suspension cultures that are useful as sources of recipient

cells. It is contemplated that alternating callus between different culture conditions may be beneficial in enriching for recipient cells within a culture. For example, it is proposed that cells may be cultured in suspension culture, but transferred to solid medium at regular intervals. After a period of growth on solid medium cells can be manually selected for return to liquid culture medium. It is proposed that by repeating this sequence of transfers to fresh culture medium it is possible to enrich for recipient cells. It also is contemplated that passing cell cultures through a 1.9 mm sieve is useful in maintaining the friability of a callus or suspension culture and may be beneficial in enriching for transformable cells.

#### **D. Cryopreservation Methods**

Cryopreservation is important because it allows one to maintain and preserve a known transformable cell culture for future use, while eliminating the cumulative detrimental effects associated with extended culture periods.

Cell suspensions and callus were cryopreserved using modifications of methods previously reported (Finkle, 1985; Withers & King, 1979). The cryopreservation protocol comprised adding a pre-cooled (0°C) concentrated cryoprotectant mixture stepwise over a period of one to two hours to pre-cooled (0°C) cells. The mixture was maintained at 0°C throughout this period. The volume of added cryoprotectant was equal to the initial volume of the cell suspension (1:1 addition), and the final concentration of cryoprotectant additives was 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 MW), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at 0°C for 30 minutes, during which time the cell suspension/cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.5°C/minute to -8°C and held at this temperature for ice nucleation.

Once extracellular ice formation had been visually confirmed, the tubes were cooled at 0.5 °C/minute from -8°C to -35°C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout the cell clusters). At this point, the cells had lost the majority of their osmotic volume (*i.e.*, there is little free water left in the cells), and they could

be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in the cells in conjunction with the rapid cooling rates from -35°C to -196°C prevented large organized ice crystals from forming in the cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to the point where long-term storage should not be detrimental.

Thawing of the extracellular solution was accomplished by removing the cryo-tube from liquid nitrogen and swirling it in sterile 42°C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter, resting on a layer of BMS cells (the feeder layer which provided a nurse effect during recovery). The cryoprotectant solution is removed by pipetting. Culture medium comprised a callus proliferation medium with increased osmotic strength. Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to the recovering cells. Once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. If initiation of a suspension culture was desired, the cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks). Alternatively, cells were cultured on solid callus proliferation medium. After the culture was reestablished in liquid (within 1 to 2 additional weeks), it was used for transformation experiments. When desired, previously cryopreserved cultures may be frozen again for storage.

## **VII. Production and Characterization of Stably Transformed Plants**

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. As mentioned herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population

by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

#### A. Selection

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphotransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Using the techniques disclosed herein, greater than 40% of bombarded embryos may yield transformants.

One herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS, which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced *in vitro*. Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, PCT Publication WO 97/04103. The best

characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT Publication WO 97/04103). Furthermore, a naturally occurring glyphosate resistant EPSPS may be used, *e.g.*, the CP4 gene isolated from *Agrobacterium* encodes a glyphosate resistant EPSPS (U.S. Patent No. 5,627,061).

Another example of a herbicide which constitutes a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato, potato (De Block *et al.*, 1987) *Brassica* (De Block *et al.*, 1989) and maize (U.S. Patent No. 5,550,318). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, bombarded tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3



mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility in the practice of the invention. Tissue can be placed on any porous, inert, solid or semi-solid support for bombardment, including but not limited to filters and solid culture medium. Bialaphos and glyphosate are provided as  
5 examples of agents suitable for selection of transformants, but the technique of this invention is not limited to them.

It further is contemplated that the herbicide dalapon, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid  
10 dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, 1992; U.S. Patent No. 5,780,708).

Alternatively, a gene encoding anthranilate synthase, which confers resistance to certain  
15 amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Patent No. 5,508,468 and PCT Publication WO 97/26366.

An example of a screenable marker trait is the red pigment produced under the control of  
20 the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media. The R-locus is useful for selection of transformants from bombarded immature embryos. In a similar fashion, the introduction of the C1 and B genes will  
25 result in pigmented cells and/or tissues.

The enzyme luciferase may be used as a screenable marker in the context of the present invention. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter),  
30 by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon

counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another screenable marker which may be used in a similar fashion is the gene coding for green fluorescent protein.

It further is contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types. This may be efficiently achieved using a gene fusion between a selectable marker gene and a screenable marker gene, for example, between an NPTII gene and a GFP gene.

#### **B. Regeneration and Seed Production**

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified (see Table 7) by including further substances such as growth regulators. A preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and like ways has been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for

regeneration, at least 2 weeks, then transferred to media conducive to maturation of embryoids. Cultures are transferred every 2 weeks on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

5 The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soilless plant growth mix, and hardened off, *e.g.*, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO<sub>2</sub>, and 25-250 microeinsteins m<sup>-2</sup> s<sup>-1</sup> of light, prior to transfer to a greenhouse or growth chamber for maturation. Plants are preferably matured either in a growth chamber or greenhouse. Plants are regenerated from about 10 6 wk to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes and Plant Cons. Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, 15 they may be transferred to a greenhouse for further growth and testing.

Note, however, that seeds on transformed plants may occasionally require embryo rescue due to cessation of seed development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected seeds 10-20 days post-pollination and 20 cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that may be cultured for 1 wk on media containing the above ingredients along with 10<sup>-5</sup>M abscisic acid and then transferred to growth regulator-free medium for germination.

25 Progeny may be recovered from transformed plants and tested for expression of the exogenous expressible gene by localized application of an appropriate substrate to plant parts such as leaves. In the case of *bar* transformed plants, it was found that transformed parental plants (R<sub>0</sub>) and their progeny of any generation tested exhibited no bialaphos-related necrosis 30 after localized application of the herbicide Basta to leaves, if there was functional PAT activity in

25094423.1

the plants as assessed by an *in vitro* enzymatic assay. All PAT positive progeny tested contained *bar*, confirming that the presence of the enzyme and the resistance to bialaphos were associated with the transmission through the germline of the marker gene.

### 5            C. Characterization

To confirm the presence of the exogenous DNA or “transgene(s)” in the regenerating plants, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays, such as Southern and Northern blotting and PCR; “biochemical” assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and  
10 Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

#### 1. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from callus cell lines or any plant parts to determine the  
15 presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.

The presence of DNA elements introduced through the methods of this invention may be  
20 determined by polymerase chain reaction (PCR). Using this technique discrete fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. Typically, DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR analysis. In addition,  
25 it is not possible using PCR techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR, *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR, *e.g.*, the presence of a gene.

Both PCR and Southern hybridization techniques can be used to demonstrate transmission of a transgene to progeny. In most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer *et al.*, 1992) indicating stable inheritance of the transgene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR™ techniques also may be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR™ techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species also can be

determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

## 2. Gene Expression

While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and  $^{14}\text{C}$ -acetyl CoA or for anthranilate synthase activity by following an increase in fluorescence as anthranilate is produced, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

#### **D. Event Specific Transgene Assays**

Southern blotting, PCR and RT-PCR techniques can be used to identify the presence or absence of a given transgene but, depending upon experimental design, may not specifically and uniquely identify identical or related transgene constructs located at different insertion points within the recipient genome. To more precisely characterize the presence of transgenic material in a transformed plant, one skilled in the art could identify the point of insertion of the transgene and, using the sequence of the recipient genome flanking the transgene, develop an assay that specifically and uniquely identifies a particular insertion event. Many methods can be used to determine the point of insertion such as, but not limited to, Genome Walker™ technology (CLONTECH, Palo Alto, CA), Vectorette™ technology (Sigma, St. Louis, MO), restriction site oligonucleotide PCR (Sarkar et al., 1993; Weber et al., 1998), uneven PCR (Chen and Wu, 1997) and generation of genomic DNA clones containing the transgene of interest in a vector such as, but not limited to, lambda phage.

Once the sequence of the genomic DNA directly adjacent to the transgenic insert on either or both sides has been determined, one skilled in the art can develop an assay to specifically and uniquely identify the insertion event. For example, two oligonucleotide primers can be designed, one wholly contained within the transgene and one wholly contained within the flanking sequence, which can be used together with the PCR technique to generate a PCR product unique to the inserted transgene. In one embodiment, the two oligonucleotide primers for use in PCR could be

designed such that one primer is complementary to sequences in both the transgene and adjacent flanking sequence such that said primer spans the junction of the insertion site while the second primer could be homologous to sequences contained wholly within the transgene. In another embodiment, the two oligonucleotide primers for use in PCR could be designed such that one primer is complementary to sequences in both the transgene and adjacent flanking sequence such that said primer spans the junction of the insertion site while the second primer could be homologous to sequences contained wholly within the genomic sequence adjacent to the insertion site. Confirmation of the PCR reaction may be monitored by, but not limited to, size analysis on gel electrophoresis, sequence analysis, hybridization of the PCR product to a specific radiolabeled DNA or RNA probe or to a molecular beacon (Tyagi and Kramer, 1996), or use of the primers in conjugation with a TaqMan<sup>TM</sup> probe and technology (PerkinElmer Corporation, Wellesley, MA).

#### **VIII. Site Specific Integration or Excision of Transgenes**

It is specifically contemplated by the inventors that one could employ techniques for the site-specific integration or excision of transformation constructs prepared in accordance with the instant invention. An advantage of site-specific integration or excision is that it can be used to overcome problems associated with conventional transformation techniques, in which transformation constructs typically randomly integrate into a host genome and multiple copies of a construct may integrate. This random insertion of introduced DNA into the genome of host cells can be detrimental to the cell if the foreign DNA inserts into an essential gene. In addition, the expression of a transgene may be influenced by "position effects" caused by the surrounding genomic DNA. Further, because of difficulties associated with plants possessing multiple transgene copies, including gene silencing, recombination and unpredictable inheritance, it is typically desirable to control the copy number of the inserted DNA, often only desiring the insertion of a single copy of the DNA sequence.



Site-specific integration can be achieved in plants by means of homologous recombination (see, for example, U.S. Patent No. 5,527,695, specifically incorporated herein by reference in its entirety). Homologous recombination is a reaction between any pair of DNA sequences having a similar sequence of nucleotides, where the two sequences interact (recombine) to form a new recombinant DNA species. The frequency of homologous recombination increases as the length of the shared nucleotide DNA sequences increases, and is higher with linearized plasmid molecules than with circularized plasmid molecules. Homologous recombination can occur between two DNA sequences that are less than identical, but the recombination frequency declines as the divergence between the two sequences increases.

Introduced DNA sequences can be targeted via homologous recombination by linking a DNA molecule of interest to sequences sharing homology with endogenous sequences of the host cell. Once the DNA enters the cell, the two homologous sequences can interact to insert the introduced DNA at the site where the homologous genomic DNA sequences were located. Therefore, the choice of homologous sequences contained on the introduced DNA will determine the site where the introduced DNA is integrated via homologous recombination. For example, if the DNA sequence of interest is linked to DNA sequences sharing homology to a single copy gene of a host plant cell, the DNA sequence of interest will be inserted via homologous recombination at only that single specific site. However, if the DNA sequence of interest is linked to DNA sequences sharing homology to a multicopy gene of the host eukaryotic cell, then the DNA sequence of interest can be inserted via homologous recombination at each of the specific sites where a copy of the gene is located.

DNA can be inserted into the host genome by a homologous recombination reaction involving either a single reciprocal recombination (resulting in the insertion of the entire length of the introduced DNA) or through a double reciprocal recombination (resulting in the insertion of only the DNA located between the two recombination events). For example, if one wishes to insert a foreign gene into the genomic site where a selected gene is located, the introduced DNA should contain sequences homologous to the selected gene. A single homologous recombination event would then result in the entire introduced DNA sequence being inserted into the selected

gene. Alternatively, a double recombination event can be achieved by flanking each end of the DNA sequence of interest (the sequence intended to be inserted into the genome) with DNA sequences homologous to the selected gene. A homologous recombination event involving each of the homologous flanking regions will result in the insertion of the foreign DNA. Thus only those DNA sequences located between the two regions sharing genomic homology become integrated into the genome.

Although introduced sequences can be targeted for insertion into a specific genomic site via homologous recombination, in higher eukaryotes homologous recombination is a relatively rare event compared to random insertion events. In plant cells, foreign DNA molecules find homologous sequences in the cell's genome and recombine at a frequency of approximately  $0.5-4.2 \times 10^{-4}$ . Thus any transformed cell that contains an introduced DNA sequence integrated via homologous recombination will also likely contain numerous copies of randomly integrated introduced DNA sequences. Therefore, to maintain control over the copy number and the location of the inserted DNA, these randomly inserted DNA sequences can be removed. One manner of removing these random insertions is to utilize a site-specific recombinase system (U.S. Patent No. 5,527,695).

A number of different site specific recombinase systems could be employed in accordance with the instant invention, including, but not limited to, the Cre/lox system of bacteriophage P1 (U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety), the FLP/FRT system of yeast (Golic and Lindquist, 1989), the Gin recombinase of phage Mu (Maeser *et al.*, 1991), the Pin recombinase of *E. coli* (Enomoto *et al.*, 1983), and the R/RS system of the pSR1 plasmid (Araki *et al.*, 1992). The bacteriophage P1 Cre/lox and the yeast FLP/FRT systems constitute two particularly useful systems for site specific integration or excision of transgenes. In these systems, a recombinase (Cre or FLP) will interact specifically with its respective site-specific recombination sequence (lox or FRT, respectively) to invert or excise the intervening sequences. The sequence for each of these two systems is relatively short (34 bp for lox and 47 bp for FRT) and therefore, convenient for use with transformation vectors.

The FLP/FRT recombinase system has been demonstrated to function efficiently in plant cells. Experiments on the performance of the FLP/FRT system in both maize and rice protoplasts indicate that FRT site structure, and amount of the FLP protein present, affects excision activity. In general, short incomplete FRT sites leads to higher accumulation of excision products than the complete full-length FRT sites. The systems can catalyze both intra- and intermolecular reactions in maize protoplasts, indicating its utility for DNA excision as well as integration reactions. The recombination reaction is reversible and this reversibility can compromise the efficiency of the reaction in each direction. Altering the structure of the site-specific recombination sequences is one approach to remedying this situation. The site-specific recombination sequence can be mutated in a manner that the product of the recombination reaction is no longer recognized as a substrate for the reverse reaction, thereby stabilizing the integration or excision event.

In the Cre-lox system, discovered in bacteriophage P1, recombination between lox sites occurs in the presence of the Cre recombinase (see, *e.g.*, U.S. Patent No. 5,658,772, specifically incorporated herein by reference in its entirety). This system has been utilized to excise a gene located between two lox sites which had been introduced into a yeast genome (Sauer, 1987). Cre was expressed from an inducible yeast GAL1 promoter and this Cre gene was located on an autonomously replicating yeast vector.

Since the lox site is an asymmetrical nucleotide sequence, lox sites on the same DNA molecule can have the same or opposite orientation with respect to each other. Recombination between lox sites in the same orientation results in a deletion of the DNA segment located between the two lox sites and a connection between the resulting ends of the original DNA molecule. The deleted DNA segment forms a circular molecule of DNA. The original DNA molecule and the resulting circular molecule each contain a single lox site. Recombination between lox sites in opposite orientations on the same DNA molecule result in an inversion of the nucleotide sequence of the DNA segment located between the two lox sites. In addition, reciprocal exchange of DNA segments proximate to lox sites located on two different DNA molecules can occur. All of these recombination events are catalyzed by the product of the Cre coding region.

## **IX. Deletion of Sequences Located within the Transgenic Insert**

During the transformation process it is often necessary to include ancillary sequences, such as selectable marker or reporter genes, for tracking the presence or absence of a desired trait gene transformed into the plant on the DNA construct. Such ancillary sequences often do not contribute to the desired trait or characteristic conferred by the phenotypic trait gene. Homologous recombination is a method by which introduced sequences may be selectively deleted in transgenic plants.

It is known that homologous recombination results in genetic rearrangements of transgenes in plants. Repeated DNA sequences have been shown to lead to deletion of a flanked sequence in various dicot species, *e.g. Arabidopsis thaliana* (Swoboda *et al.*, 1994; Jelesko *et al.*, 1999), *Brassica napus* (Gal *et al.*, 1991; Swoboda *et al.*, 1993) and *Nicotiana tabacum* (Peterhans *et al.*, 1990; Zubko *et al.*, 2000). One of the most widely held models for homologous recombination is the double-strand break repair (DSBR) model (Szostak *et al.*, 1983).

Deletion of sequences by homologous recombination relies upon directly repeated DNA sequences positioned about the region to be excised in which the repeated DNA sequences direct excision utilizing native cellular recombination mechanisms. The first fertile transgenic plants are crossed to produce either hybrid or inbred progeny plants, and from those progeny plants, one or more second fertile transgenic plants are selected which contain a second DNA sequence that has been altered by recombination, preferably resulting in the deletion of the ancillary sequence. The first fertile plant can be either hemizygous or homozygous for the DNA sequence containing the directly repeated DNA which will drive the recombination event.

The directly repeated sequences are located 5' and 3' to the target sequence in the transgene. As a result of the recombination event, the transgene target sequence may be deleted, amplified or otherwise modified within the plant genome. In the preferred embodiment, a deletion of the target sequence flanked by the directly repeated sequence will result.

## **X. Breeding Plants of the Invention**

In addition to direct transformation of a particular plant genotype with a construct prepared according to the current invention, transgenic plants may be made by crossing a plant having a construct of the invention to a second plant lacking the construct. For example, a selected coding region operably linked to a GS<sub>1-2</sub> promoter can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the current invention not only encompasses a plant directly regenerated from cells which have been transformed in accordance with the current invention, but also the progeny of such plants. As used herein the term “progeny” denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a construct prepared in accordance with the invention. “Crossing” a plant to provide a plant line having one or more added transgenes relative to a starting plant line, as disclosed herein, is defined as the techniques that result in a transgene of the invention being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene of the invention. To achieve this one could, for example, perform the following steps:

- (a) plant seeds of the first (starting line) and second (donor plant line that comprises a transgene of the invention) parent plants;
- (b) grow the seeds of the first and second parent plants into plants that bear flowers;
- (c) pollinate a flower from the first parent plant with pollen from the second parent plant; and
- (d) harvest seeds produced on the parent plant bearing the fertilized flower.

Backcrossing is herein defined as the process including the steps of:

- (a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking said desired gene, DNA sequence or element;
- (b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;
- (c) crossing the progeny plant to a plant of the second genotype; and

(d) repeating steps (b) and (c) for the purpose of transferring said desired gene, DNA sequence or element from a plant of a first genotype to a plant of a second genotype.

Introgession of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking said desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

## XI. Definitions

**Genetic Transformation:** A process of introducing a DNA sequence or construct (*e.g.*, a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

**Exogenous gene:** A gene which is not normally present in a given host genome in the exogenous gene's present form. In this respect, the gene itself may be native to the host genome, however, the exogenous gene will comprise the native gene altered by the addition or deletion of one or more different regulatory elements.

**Expression:** The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

**Expression cassette:** A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred expression cassettes will comprise all of the genetic elements necessary to direct the expression of a selected gene. Expression cassettes prepared in accordance with the instant invention will include a maize cytoplasmic glutamine synthetase GS<sub>1-2</sub> promoter.

**Expression vector:** A vector comprising at least one expression cassette.

**Obtaining:** When used in conjunction with a transgenic plant cell or transgenic plant, obtaining means either transforming a non-transgenic plant cell or plant to create the transgenic plant cell or plant, or planting transgenic plant seed to produce the transgenic plant cell or plant.

**Progeny:** Any subsequent generation, including the seeds and plants therefrom, which is  
5 derived from a particular parental plant or set of parental plants.

**Promoter:** A recognition site on a DNA sequence or group of DNA sequences that provides an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

**R<sub>0</sub> Transgenic Plant:** A plant which has been directly transformed with a selected DNA  
10 or has been regenerated from a cell or cell cluster which has been transformed with a selected DNA.

**Regeneration:** The process of growing a plant from a plant cell (*e.g.*, plant protoplast, callus or explant).

**Selected DNA:** A DNA segment which one desires to introduce into a plant genome by  
15 genetic transformation.

**Selected Gene:** A gene which one desires to have expressed in a transgenic plant, plant cell or plant part. A selected gene may be native or foreign to a host genome, but where the selected gene is present in the host genome, will include one or more regulatory or functional elements which differ from native copies of the gene.

**Transformation construct:** A chimeric DNA molecule which is designed for  
20 introduction into a host genome by genetic transformation. Preferred transformation constructs will comprise all of the genetic elements necessary to direct the expression of one or more exogenous genes. Transformation constructs prepared in accordance with the instant invention will include a maize GS<sub>1-2</sub> promoter. In particular embodiments of the instant invention, it may be  
25 desirable to introduce a transformation construct into a host cell in the form of an expression cassette.

**Transformed cell:** A cell the DNA complement of which has been altered by the introduction of an exogenous DNA molecule into that cell.

**Transgene:** A segment of DNA which has been incorporated into a host genome or is  
30 capable of autonomous replication in a host cell and is capable of causing the expression of one or

25094423.1

more cellular products. Exemplary transgenes will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant. Transgenes may be directly introduced into a plant by genetic transformation, or may be inherited from a plant of any previous generation which was transformed with the DNA segment.

5       **Transgenic plant:** A plant or progeny plant of any subsequent generation derived therefrom, wherein the DNA of the plant or progeny thereof contains an introduced exogenous DNA segment not originally present in a non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the “exogenous” gene has been altered in order to alter the level or pattern of expression  
10 of the gene.

**Transit peptide:** A polypeptide sequence which is capable of directing a polypeptide to a particular organelle or other location within a cell.

**Vector:** A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment.  
15 A plasmid is an exemplary vector.

## **XII. Examples**

      The following examples are included to illustrate preferred embodiments of the invention.  
20 It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or  
25 similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended  
30 claims.



The current inventors have demonstrated the utility of a novel maize promoter, designated the *Zea mays* cytoplasmic, pedicel-specific glutamine synthetase (GS<sub>1-2</sub>) promoter, in transgenic plants, particularly maize. The GS<sub>1-2</sub> promoter was isolated from maize 01LBH2 genomic DNA by inverse PCR. When operably linked to the *uidA* reporter gene (Jefferson *et al.*, 1986) with an intron 1 from the rice actin 1 gene (McElroy *et al.*, 1990) and a pin II 3' UTR (Graham *et al.*, 1986). The promoter of the current invention was shown to be functionally active in the young cob, at the point of silk attachment in the developing kernel of unpollinated maize, and in the pedicel tissue, endosperm basal cell layer and cob vasculature of maize kernels post-pollination.

## EXAMPLE 1

### Isolation of a *Zea mays* Cytoplasmic, Pedicel-Specific Glutamine Synthetase GS<sub>1-2</sub> Promoter

Inverse PCR was used as described herein to isolate the maize GS<sub>1-2</sub> promoter from *Zea mays* 01IBH2 (a DeKalb proprietary inbred line) genomic DNA. Approximately 1 µg of maize genomic DNA was digested with PstI restriction enzyme according to manufacturer's recommendations (Roche Molecular Biochemicals, Indianapolis IN). Following an overnight digestion at 37°C, the digestion reaction was purified using an Amicon Micropure-EZ kit and the purified, digested DNA was then concentrated to a final volume of approximately 8 µl using an Amicon Microcon apparatus, both used as per manufacturer's recommendations (Millipore Corporation, Bedford MA).

Approximately 125 ng (1 µl) of the digested genomic maize DNA was then self-ligated in the presence of T<sub>4</sub> DNA ligase, following the procedure of a Roche Rapid DNA ligation kit (Roche Molecular Biochemicals, Indianapolis IN). The ligation was carried out overnight, approximately 18 hours, at 16°C. The completed ligation reaction was heat treated at 70°C for 15 minutes, diluted by the addition of 70 µl of distilled, deionized water and stored at 4°C.

Oligonucleotide primers for a primary inverse PCR reaction were designed based upon the maize glutamine synthetase GS<sub>1-2</sub> sequence disclosed by Li *et al.*, (1993; accession number X65927). One skilled in the art would realize that other primers could be designed using this

sequence to obtain similar results. Primers as239 (SEQ ID NO:1; Figure 1) and s350 (SEQ ID NO:2; Figure 1) comprised the following sequences:

as239 5' TCA TCA ACA GGT CCG GAC AG 3'

s350 5' GGA AGG GGC AGA ACA TAC TG 3'

- 5 One µl of the diluted ligation reaction was used as the DNA template in the primary PCR reaction. The reaction comprised primers as239 (SEQ ID NO:1) and s350 (SEQ ID NO:2) in a mixture containing Buffer 3, nucleotides and thermostable DNA polymerase according to the conditions outlined by an Expand Long PCR kit (Roche Molecular Biochemicals, Indianapolis IN). A 7-step PCR program was utilized:

- 10 1. 94<sup>0</sup>C for 25 seconds  
2. 72<sup>0</sup>C for 3 minutes  
3. go to step 1 for 7 cycles  
4. 94<sup>0</sup>C for 25 seconds  
5. 67<sup>0</sup>C for 3 minutes  
15 6. go to step 4 for 32 cycles  
7. 67<sup>0</sup>C for 7 minutes

Primers for the secondary inverse PCR reaction were designed based upon the maize glutamine synthetase GS<sub>1,2</sub> sequence disclosed by Li *et al.*, (1993; accession number X65927).

- 20 The second set of primers were “nested” primers, that is, they hybridize to the glutamine synthetase GS<sub>1,2</sub> sequence at locations contained wholly within the PCR product produced in the primary PCR reaction. One skilled in the art would realize that other nested primers, such as primers that overlap with the primary PCR primers, could be designed using this sequence to obtain similar results. Primers as122 (SEQ ID NO:3; Figure 1) and s400 (SEQ ID NO:4; Figure  
25 1) comprised the following sequences:

as122 5' AGG TCG GAG AGC AGA GCC AT 3'

s400 5' GAG CCG ATC CCG AGC AAC AA 3'

One µl of the primary PCR reaction was diluted into 70 ul of distilled, deionized water and used as template DNA in the secondary PCR reaction. The reaction comprised primers as122 (SEQ ID

- 30 NO:3) and s400 (SEQ ID NO:4) in a mixture containing Buffer 3, nucleotides and thermostable  
25094423.1

DNA polymerase according to the conditions outlined by the Expand Long PCR kit (Roche Molecular Biochemicals, Indianapolis IN). A 5-step PCR program was utilized:

1. 95°C for 1 minute
2. 95°C for 20 secnds
- 5 3. 68°C for 3 minutes
4. go to step 2 for 30 cycles
5. 68°C for 7 minutes

An aliquot of the secondary PCR reaction was separated on an agarose gel, visualized with ethidium bromide stain and was shown to have produced a single band of PCR product of approximately 2.7 kilobases (Kb) in size.

An aliquot of the secondary PCR reaction was ligated into the pGEM-T-Easy vector according to the manufacturer's recommendations (Promega, Madison WI). An aliquot of the ligation reaction was used to transform competent DH5α *E. coli* cells which were plated onto solid media with the appropriate supplements to allow for ampicillin resistance and blue/white colony selection. In this type of selection, bacterial cells transformed with plasmids containing an inserted fragment of DNA are typically white in color, while cells containing plasmids that do not have additional DNA are typically blue in color. Several white colonies were selected for further analysis. One of skill in the art would realize that alternate standard molecular biology methods may be employed to clone a desired PCR DNA fragment (Sambrook and Russell, 2001; Ausubel *et al.*, 2001).

Bacterial cells from the white colonies were mixed with 10-50 µl of water, preferably 20-40 µl of water and most preferably, 30 µl of water, heated to 100°C for approximately 3 minutes and 1 µl of the slurry was used for PCR. The reaction comprised primer as122 (SEQ ID NO:3) and primer s400 (SEQ ID NO:4) in a mixture containing Buffer 3, nucleotides and thermostable DNA polymerase according to the conditions outlined by the Expand Long PCR kit (Roche Molecular Biochemicals, Indianapolis, IN). A 5-step PCR program was utilized:

1. 95°C for 1 minute
- 30 2. 95°C for 20 secnds

3. 68<sup>0</sup>C for 3 minutes
4. go to step 2 for 30 cycles
5. 68<sup>0</sup>C for 7 minutes

An aliquot of the PCR reaction was separated on an agarose gel, visualized with ethidium bromide stain and was shown to have produced a single band of PCR product approximately 2.7 kilobases (Kb) in size.

To further confirm the identity of the cloned fragment as being associated with GS<sub>1-2</sub>, it was desired to design a new oligonucleotide primer specific to the GS<sub>1-2</sub> 5' untranslated region.

Using BLAST analysis against public sequences from a variety of organisms including corn (Altschul *et al.*, 1997) it was determined that the terminal 66 base pairs at the 5' end of the untranslated region of GS<sub>1-2</sub> as reported by Li *et al.*, (1993) were unique to the GS<sub>1-2</sub> gene from maize and showed little to no significant homology to other sequences in the databases. Thus, a primer may be designed to hybridize to only to the GS<sub>1-2</sub> 5' untranslated region, and that would not hybridize with sequences reported for the five other maize GS genes. Primer s1 was designed to this unique region and comprised the following sequence (SEQ ID NO:5; Figure 1):

s1 5' CGA AAG CAC ACA CGG ATC AA 3'

The PCR reaction comprised primers as122 (SEQ ID NO:3) and s1 (SEQ ID NO:5) in a mixture containing Buffer 3, nucleotides and thermostable DNA polymerase according to the conditions outlined by the Expand Long PCR kit (Roche Molecular Biochemicals, Indianapolis IN). A 5-step PCR program was utilized:

1. 95<sup>0</sup>C for 1 minute
2. 95<sup>0</sup>C for 20 seocnds
3. 68<sup>0</sup>C for 3 minutes
4. go to step 2 for 30 cycles
5. 68<sup>0</sup>C for 7 minutes

An aliquot of the PCR reaction was separated on an agarose gel, visualized with ethidium bromide stain and was shown to have produced a single band of PCR product approximately 2.6 kilobases (Kb) in size.

The cloned PCR fragment was sequenced using SP<sub>6</sub> and T<sub>7</sub> primers hybridizing to the pGEM vector (Promega, Madison WI) as well as a series of primers which were designed to hybridize to internal sequences (SEQ ID NOS:6-17). The cloned PCR fragment was determined to be 2670 base pairs in length, 121 based pairs of which were found to overlap with 121 base pairs in the 5' untranslated region of the GS<sub>1-2</sub> gene reported by Li *et al.*, (1993; accession number X65927) and as shown by sequence alignment using Sequencher Software (version 4.0.5, Gene Codes Corporation, Ann Arbor, MI). A BLAST analysis of a 2.55 Kb subfragment of the putative GS<sub>1-2</sub> promoter (described in Example 2) showed that sequences from about base pair (bp) 100 to about bp 157, and from about bp 268 to about bp 400, showed homology to GS<sub>1-2</sub> cDNA sequence (accession number X65927; Li *et al.*, 1993). Further BLAST analysis using default parameters did not reveal homology with any other sequence in the GenBank database. These data suggest that the cloned fragment of DNA produced by inverse PCR utilizing primers designed against a maize GS<sub>1-2</sub> sequence comprises the sequence for the maize GS<sub>1-2</sub> promoter.

## EXAMPLE 2

### Construction of GS<sub>1-2</sub> Promoter Containing Transformation Vectors

This example describes the construction of vector pMON65159. The pGEM vector comprising the putative GS<sub>1-2</sub> promoter fragment was digested with NcoI and PvuI (Roche Molecular Biochemicals, Indianapolis IN) which allowed removal of approximately 2.55 Kb of the cloned 2.7 Kb insert. The ends of the digested DNA were made blunt using the Stratagene Klenow Fill-In Kit (Stratagene, La Jolla CA). The products of the digestion were separated on an agarose gel and a slice containing the 2.55 Kb fragment with the putative GS<sub>1-2</sub> promoter was removed from the gel. The DNA was purified from the agarose gel using a GenElute Agarose Spin column (Sigma Chemical Co., St. Louis, MO) as per manufacturer's recommendations.

Vector pGUS33, comprising the *uidA* screenable marker gene (Jefferson *et al.*, 1986), intron 1 from the rice actin 1 gene (McElroy *et al.*, 1990) and *pinII* 3' UTR (Graham *et al.*, 1986), as well as the 35S promoter, (Odell *et al.*, 1985), the NPT II selectable marker gene (Potrykus *et al.*, 1985) and *nos* 3' UTR (Bevan *et al.*, 1983), was digested with XhoI and BstEII (Roche Molecular Biochemicals, Indianapolis IN). The ends digested DNA were made blunt

using the Stratagene Klenow Fill-In Kit (Stratagene, La Jolla CA). The filled-in ends of the pGUS33 backbone fragment, as well as other fragments from the digestion, were also dephosphorylated with calf alkaline intestinal phosphatase (Roche Molecular Biochemicals, Indianapolis IN). The approximately 7.8 Kb backbone fragment containing the *uidA* gene, intron and *pinII* 3'UTR and the 35S promoter, NPT II gene and *nos* 3' UTR, was isolated from an agarose gel slice using a GenElute Agarose Spin column (Sigma Chemical Co., St. Louis, MO) as per manufacturer's recommendations. The GS<sub>1-2</sub> promoter DNA fragment and the pGUS33 backbone fragment were ligated together using a Roche Rapid DNA ligation kit (Roche Molecular Biochemicals, Indianapolis IN) and ligation products transformed into competent *E. coli* cells.

Transformed *E. coli* cells were plated upon solid medium supplemented with ampicillin and resistant colonies selected. Resistant colonies were selected and grown overnight in liquid media supplemented with ampicillin as per standard molecular procedures (see for example, Ausubel *et al.*, 2001; Sambrook and Russell, 2001). Plasmid DNA was isolated using a Qiagen midi-prep protocol (QIAGEN, Inc., Valencia, CA).

DNA comprising the putative GS<sub>1-2</sub> promoter and GUS reporter gene was isolated for microprojectile bombardment. Plasmid pMON65159 was digested with NotI and SgfI as per manufacturer's recommendations (Roche Molecular Biochemicals, Indianapolis IN) and separated on a agarose gel. The approximately 7.76 Kb fragment containing the maize cytoplasmic GS<sub>1-2</sub> promoter/rice actin intron/GUS/*pinII* 3'UTR and 35S promoter/NPT II/*nos* 3' UTR, was excised from the agarose gel and purified using the GenElute<sup>TM</sup> agarose spin column as per manufacturer's suggested protocol (Sigma, St. Louis, MO).

### EXAMPLE 3

#### Preparation of Microprojectiles

Microprojectiles were prepared for use with the electric discharge particle acceleration gene delivery device (U.S. Patent No. 5,015,580) by suspending 10 mg of 0.6 µm gold particles (BioRad) in 50 µl buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0). About 10 to 1000 ng,

preferably about 50 to 500 ng, more preferably 50 to 250 ng, and most preferably, about 150 ng of cassette DNA isolated from pMON65159 was added to the suspension of gold particles and gently vortexed for about five seconds.

5           Seventy five  $\mu$ l of 0.1M spermidine was added and the solution vortexed gently for about 5 seconds. Seventy five  $\mu$ l of a 25% solution of polyethylene glycol (3000-4000 molecular weight, American Type Culture Collection) was added and the solution was gently vortexed for five seconds. Seventy five  $\mu$ l of 2.5 M  $\text{CaCl}_2$  was added and the solution vortexed for five seconds. Following the addition of  $\text{CaCl}_2$ , the solution was incubated at room temperature for 10  
10 to 15 minutes. The suspension was subsequently centrifuged for 20 seconds at 12,000 rpm (Sorval MC-12V centrifuge) and the supernatant discarded. The gold particle/DNA pellet was washed twice with one ml 100% ethanol and resuspended to a total volume of 10 ml in 100% ethanol. The gold particle/DNA preparation was stored at  $-20^\circ\text{C}$  for up to two weeks.

15           DNA was introduced into maize cells using the electric discharge particle acceleration gene delivery device (U.S. Patent No. 5,015,580). The gold particle/DNA suspension was coated on Mylar sheets (Du Pont Mylar polyester film type SMMC2, aluminum coated on one side, over coated with PVDC co-polymer on both sides, cut to 18 mm square) by dispersion of 310 to 320  
20  $\mu$ l of the gold particle/DNA suspension on a sheet. After the gold particle suspension settled for one to three minutes, excess ethanol was removed and the sheets were air dried. Microprojectile bombardment of maize tissue was conducted as described in U.S. Patent No. 5,015,580. AC voltage may be varied in the electric discharge particle delivery device. For microprojectile bombardment of Hi-II or H99 pre-cultured immature embryos, 30% to 40% of maximum voltage was preferably used. Following microprojectile bombardment, tissue was cultured in the dark at  
25  $27^\circ\text{C}$ .

#### EXAMPLE 4

##### Bombardment of H99 Immature Embryos

25094423.1

Many variations in techniques for microprojectile bombardment are well known in the art and therefore deemed useful with the current invention. Exemplary procedures for bombardment are discussed in, for example, PCT Publication No. WO 95/06128, the disclosure of which is specifically incorporated herein by reference in its entirety. Examples of target tissues which may be used with the current invention include immature embryos, Type I callus, Type II callus, Type III callus, suspension cultures and meristematic tissue (PCT Publication WO 96/04392). Some genotypes which are especially useful for maize transformation are specifically disclosed herein above, as well as in, for example, PCT Publication WO 95/06128. Preferred genotypes will be those which are readily transformable and which also may be regenerated to yield a fertile transgenic plant.

Any method for acceleration of microprojectiles may potentially be used to transform a plant cell with the current invention. A preferred method will be a gas-driven particle gun such as the DuPont Biolistics PDS1000He particle bombardment device. Exemplary particles for bombardment include those comprised of tungsten, gold, platinum, and the like. Gold particles are deemed particularly useful in the current invention, with 0.6  $\mu\text{m}$  or 0.7  $\mu\text{m}$  gold particles being preferred and 0.6  $\mu\text{m}$  particles most preferred. The most preferred particles will be DNA coated and have a mean size between 0.6  $\mu\text{m}$  and 1.0  $\mu\text{m}$ . Alternatively, particles may be allowed to settle for 2-5 minutes following precipitation of DNA onto particles. Particles are then removed from the supernatant and used for microprojectile bombardment. It is believed that the settling step enriches for a population of particles coated with DNA in which fewer aggregates of particles are present.

As disclosed herein, any DNA sequence may potentially be used for transformation. The DNA segments used for transformation will preferably include one or more selectable, secretable or screenable markers. Many examples of such are well known in the art and are specifically disclosed herein. In the case of selectable markers, selection may be in solid or liquid media. The DNA segments used will preferably also include one or more genes which confer, either individually or in combination with other sequences, a desired phenotype on the transformed



plant. Exemplary genes for transformation and the corresponding phenotypes these sequences may confer on the transformed plant are disclosed herein.

The isolated 7.76 Kb fragment containing the maize cytoplasmic GS<sub>1-2</sub> promoter/rice actin intron/GUS/*pinII* 3'UTR and 35S promoter/NPT II/*nos* 3' UTR was introduced into H99 immature embryos. Maize immature embryos (1.2 - 3.0 mm, 10-14 days post pollination) were isolated from greenhouse grown H99 plants that had been self or sib pollinated. Immature embryos were cultured on #211V medium in the dark at approximately 27°C (see Table 7 for a listing of media useful for transformation). Immature embryos were bombarded 0-6 days after isolation. Prior to bombardment, the immature embryos were transferred to 211 medium containing 12% sucrose (#211SV) for 3-6 hours. Following bombardment, carried out as described in Example 4, tissue cultures were incubated overnight and transferred to #211L medium (500mg/L paromomycin). Every 2-3 weeks, callus was transferred to fresh selection medium (211L; 500 mg/L paromomycin) and callus may be subdivided into small pieces (approximately 2-4 mm in diameter) during transfer to fresh medium. This subculture step was repeated at 2-3 week intervals for up to about 3-15 weeks post-bombardment, typically 6 to 9 weeks, with subdivision and visual selection for healthy, growing callus. Approximately 8 to 9 weeks post bombardment, the viable calli were transferred to media #217 containing 3.52 mg/L BAP (6-benzylaminopurine). Approximately one week later, the events were transferred to #127T medium in PHTYATRAYS<sup>TM</sup> and placed in the light (see Example 6).

Alternatively, immature embryos could be cultured to produce embryogenic callus that can be used for bombardment. Embryogenic callus is expanded and maintained by subculturing at 2-3 week intervals to fresh #211 medium. Prior to bombardment, embryogenic callus (subdivided in approximately 2-4 mm clumps) or, preferably cultured embryos, are transferred to 211 medium containing 12% sucrose for 3-6 hours. As described above for immature embryos, the bombed callus is transferred to medium with increasing amounts of paromomycin to select for transformed tissue.

## EXAMPLE 5

### Regeneration of fertile transgenic plants

Fertile transgenic plants were produced from transformed H99 maize cells. Transformed callus was transferred to maturation medium 217 (N6 salts, 1 mg/L thiamine-HCl, 0.5 mg/L niacin, 3.52 mg/L benzylaminopurine, 0.91 mg/L L-asparagine monohydrate, 100 mg/L myo-inositol, 0.5 g/L MES, 1.6 g/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 100 mg/L casein hydrolysate, 0.69 g/L L-proline, 20 g/L sucrose, 2 g/L GELGRO™, pH 5.8) for five to nine days in the dark at 26°-28°C, whereupon somatic embryos mature and shoot regeneration begins. Tissue was transferred to medium 127T (MS salts, 0.65 mg/L niacin, 0.125 mg/L pyridoxine-HCl, 0.125 mg/L thiamine-HCl, 0.125 mg/L Ca pantothenate, 150 mg/L L-asparagine, 100 mg/L myo-inositol, 10 g/L glucose, 20 g/L L-maltose, 100 mg/L paromomycin, 5.5 g PHYTAGAR™, pH 5.8) for shoot development. Tissue on medium 127T was cultured in the light at 400-600 lux at 26°C. Plantlets were transferred to soil about 3 to 6 weeks after transfer to 127T medium when the plantlets were about 3 inches tall and had roots. Plantlets were grown further in a growth chamber and fully matured in a greenhouse.

## EXAMPLE 6

### Analysis of Maize GS<sub>1-2</sub> Promoter Expression in Fertile Transgenic Maize

Regenerated plants from a number of independent transformation events were assayed for GUS activity by histochemical staining and were positive for *uidA* reporter gene expression as driven by the maize GS<sub>1-2</sub> promoter (Table 8). The R<sub>0</sub> plants were crossed to a proprietary inbred line (H99) or were self-pollinated, and resultant R<sub>1</sub> plant progeny, as well as progeny of subsequent generations, may be analyzed for GUS expression.

In stably transformed R<sub>0</sub> maize plants produced from the transformation procedure, the GS<sub>1-2</sub> promoter was shown to drive expression of GUS protein in the developing cob and at the point of silk attachment to the kernel in pre-pollination maize plants. No expression was observed in the leaves, roots and stems of the pre-pollination stage plants. The pre-pollination plants were collected when the cob was present and the silks had not yet emerged, preferably from about V17, when the tip of the ear may protrude from the leaf axil and the tip of the tassel

25094423.1

may be visible to about R1, when the silks have emerged, and even more preferably, from about V18, when the tassel is visible and the silks are initiating on at least a portion of the ear to about VT, when the tassel is visible and completely branched but no silks have emerged from the ear.

5 GUS expression as driven by the maize GS<sub>1-2</sub> promoter was also characterized in transformed R<sub>0</sub> plants following pollination with line H99 or after self-fertilization. The expression patterns were the same in the plants that were crossed to H99 as the plants that were self-pollinated. Tissue samples may range in age from 1 day after pollination to kernel maturity. Samples were collected at 3 days, 13 days, 20 days and 22 days post-pollination. A variety of  
10 tissues were examined for GUS expression at each of these time points including cobs, kernels, leaves, roots, stems, silks and anthers.

At 3 days post-pollination, the maize GS<sub>1-2</sub> promoter was shown to drive little to no GUS expression in the developing kernel, and no expression was observed in the leaves, roots, stems,  
15 silks and anthers. Limited expression was observed in the cob vasculature, and the developing pedicel and basal conducting cells of the endosperm (Table 8). The GUS expression observed at the point of silk attachment in the pre-pollination kernel was greatly reduced in this location on the kernel by 3 days post pollination.

At 13 days post-pollination, GUS expression as driven by the GS<sub>1-2</sub> promoter was shown  
20 to be elevated in the cob and developing kernel in comparison to the same tissues 3 days post-pollination. No expression was observed in the leaves, roots, stems, silks, point of silk attachment on the kernel and anthers of plants sampled 13 days post-pollination.

25 Twenty days post-pollination, high levels of GUS expression as directed by the maize GS<sub>1-2</sub> promoter were observed in the pedicel and endosperm basal transfer cell layer of the young kernel. Expression levels in the cob vasculature were similar to expression levels at earlier times in kernel development. Expression of GUS at the point of silk attachment to the kernel was diminished by 20 days post-pollination. GUS expression as directed by the maize GS<sub>1-2</sub> promoter  
30 was not observed in leaves, roots, silks, anthers or stems of R<sub>0</sub> plants 20 days after pollination.

25094423.1

GUS expression as driven by the maize GS<sub>1-2</sub> promoter in tissues 22 days post-pollination were similar in pattern and level as that observed for samples 20 days post-pollination.

**Table 8: GUS Expression in Maize Tissues as Driven by the Maize GS<sub>1-2</sub> Promoter**

	R <sup>1</sup>	L <sup>2</sup>	S <sup>3</sup>	SK <sup>4</sup>	A <sup>5</sup>	P <sup>6</sup>	CV <sup>7</sup>	Pd <sup>8</sup>	End <sup>9</sup>	Emb <sup>10</sup>	BCC <sup>11</sup>	SS <sup>12</sup>
Pre Poll	-	-	-	-	-	-	++	+	-	-	-	+++
3 DAP	-	-	-	-	-	-	++	++	-	-	+	+
9 DAP	-	-	-	-	-	-	++	++	-	-	++	-
13 DAP	-	-	-	-	-	-	++	+++	-	-	+++	-
20 + 22 DAP	-	-	-	-	-	-	++	++++	-	-	++++	-

(+) is lowest expression level after overnight stain

(+++++) is highest expression level after overnight stain

(-) is no GUS expression detected after overnight stain

PrePoll = pre pollination; DAP = days after pollination; 1, R=Root; 2, L=Leaf; 3, S=Stem; 4, SK=Silk; 5, A=Anther; 6, P=Pollen; 7, CV=Cob vasculature; 8, Pd=Pedicel; 9, End=Endosperm; 10, Emb=Embryo; 11, BCC=Basal conducting cells; 12, SS=Silk scar.

## EXAMPLE 7

### Transformation of Maize Using *Agrobacterium tumefaciens*

Methods of *Agrobacterium* mediated transformation of maize cells and other monocots are known (Hiei *et al.*, 1997; U.S. Patent No. 5,591,616; U.S. Patent No. 5,981,840; published EP patent application EP 0 672 752). Although various strains of *Agrobacterium* may be used (see references above), strain ABI is used preferably by the present inventors. The ABI strain of *Agrobacterium* is derived from strain A208, a C58 nopaline type strain, from which the Ti plasmid was eliminated by culture at 37°C, and further containing the modified Ti plasmid pMP90RK (Koncz and Schell, 1986). An *Agrobacterium tumefaciens* binary vector system (An *et al.*, 1998) is preferably used to transform maize. Alternative cointegrating Ti plasmid vectors have been described (Rogers *et al.*, 1988) and could be used to transform maize. A binary vector comprising one or more genes of interest may be introduced into a disarmed *Agrobacterium* strain using electroporation (Wen-jun and Forde, 1989) or triparental mating (Ditta *et al.*, 1980). A binary vector may contain a selectable marker gene, a screenable marker gene and/or one or more genes that confer a desirable phenotypic trait on the transformed plant. An exemplary binary

vector, pMON30113, is shown in FIG. 4. Other binary vectors may be used and are known to those of skill in the art.

Prior to co-culture of maize cells, *Agrobacterium* cells may be grown at 28°C in LB (DIFCO) liquid medium comprising appropriate antibiotics to select for maintenance of the modified Ti plasmid and binary vector. For example, ABI/pMON30113, may be grown in LB medium containing 50 ug/ml kanamycin to select for maintenance of the pMP90RK modified Ti plasmid and 100 ug/ml spectinomycin to select for maintenance of the binary vector pMON30113. It will be obvious to one of skill in the art to use appropriate selection agents to maintain plasmids in the host *Agrobacterium* strain. Prior to inoculation of maize cells, *Agrobacterium* cells are grown overnight at room temperature in AB medium (Chilton *et al.*, 1974) comprising appropriate antibiotics for plasmid maintenance and 200 uM acetosyringone. Immediately prior to inoculation of maize cells, *Agrobacterium* are preferably pelleted by centrifugation, washed in ½ MSVI medium (1.1 g/L GIBCO MS salts, 2 mg/L glycine, 0.5 g/L niacin, 0.5 g/L L-pyridoxine-HCl, 0.1 mg/L thiamine, 115 g/L L-proline, 10 g/L D-glucose, and 10 g/L sucrose, pH 5.4) containing 200 uM acetosyringone, and resuspended at 0.1 to 1.0 x 10<sup>9</sup> cells/ml in ½ MSPL medium (1.1 g/L GIBCO MS salts, 2 mg/L glycine, 0.5 g/L niacin, 0.5 g/L L-pyridoxine-HCl, 0.1 mg/L thiamine, 115 g/L L-proline, 26 g/L D-glucose, 68.5 g/L sucrose, pH 5.4) containing 200 uM acetosyringone. One of skill in the art may substitute other media for ½ MSPL.

Immature maize embryos are isolated as described previously. Embryos are inoculated with *Agrobacterium* 0-7 days after excision, preferably immediately after excision. Alternatively, immature embryos may be cultured for more than 7 days. For example, embryogenic callus may be initiated as described above and co-cultured with *Agrobacterium*. Preferably, immature maize embryos are excised, immersed in an *Agrobacterium* suspension in ½ MSPL medium prepared as described above and incubated at room temperature with *Agrobacterium* for 5-20 minutes.

Following inoculation embryos are transferred to one-half strength MS medium (Murashige and Skoog, 1962) containing 3.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1% D-glucose, 2% sucrose, 0.115 g/L L-proline, 0.5 mg/L thiamine-HCl, 200 uM acetosyringone,

and 20 uM silver nitrate or silver thiosulfate. Immature embryos are co-cultured with *Agrobacterium* for 1 to 3 days at 23°C in the dark. One of skill in the art may substitute other media for the described media.

5 Co-cultured embryos are transferred to medium 15AA (462 mg/L (NH<sub>4</sub>)SO<sub>4</sub>, 400 mg/L KH<sub>2</sub>PO<sub>4</sub>, 186 mg/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 166 mg/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 mg/L MnSO<sub>4</sub>·H<sub>2</sub>O, 3 mg/L H<sub>3</sub>BO<sub>3</sub>, 2 mg/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 mg/L NaMoO<sub>4</sub>·2H<sub>2</sub>O, 0.025 mg/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.025 mg/L CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.75 mg/L KI, 2.83 g/L KNO<sub>3</sub>, 0.2 mg/L niacin, 0.1 mg/L thiamine-HCl, 0.2 mg/L pyridoxine-HCl, 0.1 mg/L D-biotin, 0.1 mg/L choline chloride, 0.1 mg/L calcium  
10 pantothenate, 0.05 mg/L folic acid, 0.05 mg/L p-aminobenzoic acid, 0.05 mg/L riboflavin, 0.015 mg/L vitamin B<sub>12</sub>, 0.5 g/L casamino acids, 33.5 mg/L Na<sub>2</sub>EDTA, 1.38 g/L L-proline, 20 g/L sucrose, 10 g/L D-glucose), or MS medium containing 1.5 mg/L 2,4-D, 500 mg/L carbenicillin, 3% sucrose, 1.38 g/L L-proline and 20 uM silver nitrate or silver thiosulfate and cultured for 0 to 8 days in the dark at 27°C without selection. Culture media used for selection of transformants and regeneration of plants preferably contains 500 mg/L carbenicillin. One of skill in the art may  
15 substitute other antibiotics that control growth of *Agrobacterium*. Other culture media that support cell culture may be used alternatively. In the absence of a delay of selection (0 day culture), selection may be initiated on 25 mg/L paromomycin. Selection medium may comprise medium 211 (described above) or a variant of medium 211 in which N<sub>6</sub> salts are replaced by MS  
20 salts. After two weeks, embryogenic callus are transferred to culture medium containing 100 mg/L paromomycin and subcultured at about two week intervals. When selection is delayed following co-culture, embryos were initially cultured on medium containing 50 mg/L paromomycin followed by subsequent culture of embryogenic callus on medium containing 100-200 mg/L paromomycin. One of skill in the art will culture tissue on concentrations of  
25 paromomycin which inhibit growth of cells lacking the selectable marker gene, but a concentration on which transformed callus will proliferate. Alternatively, one may use other selectable markers to identify transformed cells. It is believed that initial culture on 25 to 50 mg/L paromocycin for about two weeks, followed by culture on 50-200 mg/L paromocycin will result in recovery of transformed callus. Transformants are recovered 6 to 8 weeks after initiation of selection. Plants

are regenerated from transformed embryogenic callus as described above for transformants recovered following microprojectile bombardment.

## EXAMPLE 8

### Introgression of Transgenes Into Elite Varieties

Backcrossing can be used to improve a starting plant. Backcrossing transfers a specific desirable trait from one source to an inbred or other plant that lacks that trait. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate gene(s) for the trait in question, for example, a construct prepared in accordance with the current invention. The progeny of this cross first are selected in the resultant progeny for the desired trait to be transferred from the non-recurrent parent, then the selected progeny are mated back to the superior recurrent parent (A). After five or more backcross generations with selection for the desired trait, the progeny are hemizygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other genes. The last backcross generation would be selfed to give progeny which are pure breeding for the gene(s) being transferred, *i.e.* one or more transformation events.

Therefore, through a series a breeding manipulations, a selected transgene may be moved from one line into an entirely different line without the need for further recombinant manipulation. Transgenes are valuable in that they typically behave genetically as any other gene and can be manipulated by breeding techniques in a manner identical to any other corn gene. Therefore, one may produce inbred plants which are true breeding for one or more transgenes. By crossing different inbred plants, one may produce a large number of different hybrids with different combinations of transgenes. In this way, plants may be produced which have the desirable agronomic properties frequently associated with hybrids ("hybrid vigor"), as well as the desirable characteristics imparted by one or more transgene(s).

## EXAMPLE 9

### Marker Assisted Selection

25094423.1

Genetic markers may be used to assist in the introgression of one or more transgenes of the invention from one genetic background into another. Marker assisted selection offers advantages relative to conventional breeding in that it can be used to avoid errors caused by phenotypic variations. Further, genetic markers may provide data regarding the relative degree of elite germplasm in the individual progeny of a particular cross. For example, when a plant with a desired trait which otherwise has a non-agronomically desirable genetic background is crossed to an elite parent, genetic markers may be used to select progeny which not only possess the trait of interest, but also have a relatively large proportion of the desired germplasm. In this way, the number of generations required to introgress one or more traits into a particular genetic background is minimized.

In the process of marker assisted breeding, DNA sequences are used to follow desirable agronomic traits in the process of plant breeding (Tanksley *et al.*, 1989). Marker assisted breeding may be undertaken as follows. Seed of plants with the desired trait are planted in soil in the greenhouse or in the field. Leaf tissue is harvested from the plant for preparation of DNA at any point in growth at which approximately one gram of leaf tissue can be removed from the plant without compromising the viability of the plant. Genomic DNA is isolated using a procedure modified from Shure *et al.* (1983). Approximately one gram of leaf tissue from a seedling is lypholyzed overnight in 15 ml polypropylene tubes. Freeze-dried tissue is ground to a powder in the tube using a glass rod. Powdered tissue is mixed thoroughly with 3 ml extraction buffer (7.0 urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 3 ml phenol/chloroform. The aqueous phase is separated by centrifugation, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate pH 5.2, and an equal volume of isopropanol. The precipitate is washed with 75% ethanol and resuspended in 100-500 µl TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

Genomic DNA is then digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran using 10X SCP (20 SCP: 2M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). The filters are prehybridized in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml denatured



salmon sperm DNA and  $^{32}\text{P}$ -labeled probe generated by random priming (Feinberg & Vogelstein, 1983). Hybridized filters are washed in 2X SCP, 1% SDS at 65°C for 30 minutes and visualized by autoradiography using Kodak XAR5 film. Genetic polymorphisms which are genetically linked to traits of interest are thereby used to predict the presence or absence of the traits of interest.

5

Those of skill in the art will recognize that there are many different ways to isolate DNA from plant tissues and that there are many different protocols for Southern hybridization that will produce identical results. Those of skill in the art will recognize that a Southern blot can be stripped of radioactive probe following autoradiography and re-probed with a different probe. In this manner one may identify each of the various transgenes that are present in the plant. Further, one of skill in the art will recognize that any type of genetic marker which is polymorphic at the region(s) of interest may be used for the purpose of identifying the relative presence or absence of a trait, and that such information may be used for marker assisted breeding.

10

Each lane of a Southern blot represents DNA isolated from one plant. Through the use of multiplicity of gene integration events as probes on the same genomic DNA blot, the integration event composition of each plant may be determined. Correlations may be established between the contributions of particular integration events to the phenotype of the plant. Only those plants that contain a desired combination of integration events may be advanced to maturity and used for pollination. DNA probes corresponding to particular transgene integration events are useful markers during the course of plant breeding to identify and combine particular integration events without having to grow the plants and assay the plants for agronomic performance.

15

20

It is expected that one or more restriction enzymes will be used to digest genomic DNA, either singly or in combinations. One of skill in the art will recognize that many different restriction enzymes will be useful and the choice of restriction enzyme will depend on the DNA sequence of the transgene integration event that is used as a probe and the DNA sequences in the genome surrounding the transgene. For a probe, one will want to use DNA or RNA sequences which will hybridize to the DNA used for transformation. One will select a restriction enzyme that produces a DNA fragment following hybridization that is identifiable as the transgene

25

30

25094423.1

integration event. Thus, particularly useful restriction enzymes will be those which reveal polymorphisms that are genetically linked to specific transgenes or traits of interest.

Polymorphisms and other sequence characteristics unique to a given genome, and useful for marker assisted selection, may be detected by methods other than Southern blotting. Simple Sequence Repeats (SSRs), also known as microsatellites or single sequence repeats, are one example of sequences that are useful as markers for identifying a particular genome or type of genome (Taramino and Tingey, 1996; Senior and Heun, 1993). SSRs are regions of the genome which are characterized by numerous dinucleotide or trinucleotide repeats, e.g., AGAGAGAG. Related but non-identical genomes often contain related but non-identical SSRs of different sizes. The genomes may thus be characterized by the length of the SSR as detected by molecular means known to those of skill in the art, such as polymerase chain reaction and size separation of the reaction products on agarose or acrylamide gel matrices.

Single Nucleotide Polymorphisms (SNPs) are other sequence variations that are useful as markers for identifying and differentiating DNA sequences (Tenaillon *et al.*, 2001). In a given genome, for example a maize genome, genes or sequences with related or identical functions in one line will have sequences that are related or identical to genes or sequences in another line. In many cases, the sequence or gene differs from line to line by a single nucleotide substitution, insertion or deletion. A gene or sequence region may contain one or several SNPs in one or several regions of the gene or sequence. Once an SNP has been identified using sequence analysis and other molecular tools, a variety of means exist for the analysis of SNPs to identify genomes. These tools include the use of PCR, sequence analysis, TaqMan™ technology (PerkinElmer Corporation, Wellesley, MA), molecular beacons and other standard molecular biology methods known to one of skill in the art.

## EXAMPLE 10

### General Methods for Assays

DNA analysis of transformed plants is performed as follows. Genomic DNA is isolated using a procedure modified from Shure *et al.*, 1983. Approximately 1 gm callus or leaf tissue is

25094423.1

ground to a fine powder in liquid nitrogen using a mortar and pestle. Powdered tissue is mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate is extracted with 4 ml phenol/ chloroform. The aqueous phase is separated by centrifugation, passed through Miracloth, and precipitated  
5 twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate is washed with 70% ethanol and resuspended in 200-500 µl TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0).

The presence of a DNA sequence in a transformed cell may be detected through the use of  
10 polymerase chain reaction (PCR). Using this technique specific fragments of DNA can be amplified and detected following agarose gel electrophoresis. For example, two hundred to 1000 ng genomic DNA is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mg/ml gelatin, 200µM each dATP, dCTP, dGTP, dTTP, 0.5µM each forward and reverse DNA primers, 20% glycerol, and 2.5 units Taq DNA polymerase. The reaction is run  
15 in a thermal cycling machine as follows: 3 minutes at 94°C, 39 repeats of the cycle 1 minute at 94 °C, 1 minute at 50°C, 30 seconds at 72°C, followed by 5 minutes at 72°C. Twenty µl of each reaction mix is run on a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours. Using this procedure, for example, one may detect the presence of the *uidA* gene. Primers for the maize GS<sub>1-2</sub> promoter can be readily prepared by one of skill in the art  
20 in light of the sequence given in SEQ ID NO:18.

A method to detect the presence of phosphinothricin acetyl transferase (PAT) involves the use of an *in vitro* enzyme reaction followed by thin layer chromatography, as described in U.S. Patent No. 5,990,890 (specifically incorporated herein by reference in its entirety). The procedure  
25 is conducted by preparing various protein extracts from homogenates of potentially transformed cells, and from control cells that have neither been transformed nor exposed to bialaphos selection, and then assaying by incubation with PPT and <sup>14</sup>C-Acetyl Coenzyme A followed by thin layer chromatography. The results of this assay provide confirmation of the expression of the *bar* gene which codes for phosphinothricin acetyl transferase (PAT).

For Southern blot analysis genomic DNA is digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Probes are labeled with  $^{32}\text{P}$  using the random priming method (Boehringer Mannheim) and purified using Quik-Sep® spin columns (Isolab Inc., Akron, OH). Filters are prehybridized at 65°C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml heparin (Chomet *et al.*, 1987) for 15 min. Filters then are hybridized overnight at 65°C in 6X SCP containing 100 µg/ml denatured salmon sperm DNA and  $^{32}\text{P}$ -labeled probe. Filters are washed in 2X SCP, 1% SDS at 65°C for 30 min. and visualized by autoradiography using Kodak XAR5 film. For rehybridization, the filters are boiled for 10 min. in distilled H<sub>2</sub>O to remove the first probe and then prehybridized as described above.

## EXAMPLE 11

### Utilization of Transgenic Crops

The ultimate goal in plant transformation is to produce plants which are useful to man. In this respect, transgenic plants created in accordance with the current invention may be used for virtually any purpose deemed of value to the grower or to the consumer. For example, one may wish to harvest seed from transgenic plants. This seed may in turn be used for a wide variety of purposes. The seed may be sold to farmers for planting in the field or may be directly used as food, either for animals or humans. Alternatively, products may be made from the seed itself. Examples of products which may be made from the seed include, oil, starch, animal or human food, pharmaceuticals, and various industrial products. The food uses of maize, in addition to human consumption of maize kernels, include both products of dry- and wet-milling industries. The principal products of maize dry milling are grits, meal and flour. The maize wet-milling industry can provide maize starch, maize syrups, and dextrose for food use. Maize oil is recovered from maize germ, which is a by-product of both dry- and wet-milling industries.

Maize, including both grain and non-grain portions of the plant, also is used extensively as livestock feed, primarily for beef cattle, dairy cattle, hogs, and poultry. Industrial uses of maize include production of ethanol, maize starch in the wet-milling industry and maize flour in the dry-



## REFERENCES

The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

- 5 U.S. Patent No. 4,237,224  
U.S. Patent No. 4,535,060  
U.S. Patent No. 4,959,317  
U.S. Patent No. 5,015,580  
U.S. Patent No. 5,134,074  
10 U.S. Patent No. 5,168,053  
U.S. Patent No. 5,188,642  
U.S. Patent No. 5,268,526  
U.S. Patent No. 5,302,523  
U.S. Patent No. 5,322,783  
15 U.S. Patent No. 5,354,855  
U.S. Patent No. 5,384,253  
U.S. Patent No. 5,391,725  
U.S. Patent No. 5,464,765  
U.S. Patent No. 5,489,520  
20 U.S. Patent No. 5,500,365  
U.S. Patent No. 5,508,184  
U.S. Patent No. 5,508,468  
U.S. Patent No. 5,510,471  
U.S. Patent No. 5,527,695  
25 U.S. Patent No. 5,538,877  
U.S. Patent No. 5,538,880  
U.S. Patent No. 5,545,818  
U.S. Patent No. 5,550,318

U.S. Patent No. 5,563,055

U.S. Patent No. 5,563,324

U.S. Patent No. 5,591,616

U.S. Patent No. 5,610,042

5 U.S. Patent No. 5,624,824

U.S. Patent No. 5,625,047

U.S. Patent No. 5,627,061

U.S. Patent No. 5,633,448

U.S. Patent No. 5,641,876

10 U.S. Patent No. 5,658,772

U.S. Patent No. 5,689,052

U.S. Patent No. 5,728,925

U.S. Patent No. 5,780,708

U.S. Patent No. 5,780,709

15 U.S. Patent No. 5,831,011

U.S. Patent No. 5,981,840

U.S. Patent No. 5,990,890

EP 0 154 204

EP 0 672 752

20 PCT Publication WO 92/17598

PCT Publication WO 95/06128

PCT Publication WO 97/04103

PCT Publication WO 96/04392

PCT Publication WO 97/26366

25 PCT Publication WO 97/41228

PCT Publication WO 98/26064

PCT Publication WO 99/58659

PCT Publication WO 00/70066

PCT Publication WO 00/70067

30

25094423.1

Abdullah *et al.*, *Biotechnology*, 4:1087, 1986.

Abel *et al.*, *Science*, 232:738-743, 1986.

Albert *et al.*, *Plant J.*, 7(4):649-659, 1995.

5 Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-3402, 1997.

An *et al.*, *Plant Molecular Biology Manual A3*:1-19, 1998.

Araki *et al.*, *J. Mol. Biol.* 225(1):25-37, 1992.

Ausubel *et al.*, *Current Protocols in Molecular Biology*, pub. John Wiley & Sons, Inc., 1987, including updates to Winter 2001.

10 Bansal *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:3654-3658, 1992.

Barkai-Golan *et al.*, *Arch. Microbiol.*, 116:119-124, 1978.

Bates, *Mol. Biotechnol.*, 2(2):135-145, 1994.

Battraw and Hall, *Theor. App. Genet.*, 82(2):161-168, 1991.

Belanger and Kriz, *Genet.*, 129:863-872, 1991.

5 Bellus, *J. Macromol. Sci. Pure Appl. Chem.*, 1(1):1355-1376, 1994.

Bernal-Lugo and Leopold, *Plant Physiol.*, 98:1207-1210, 1992.

Berzal-Herranz *et al.*, *Genes and Devel.*, 6:129-134, 1992.

Bevan *et al.*, *Nucleic Acids Research*, 11(2):369-385, 1983.

Bhattacharjee *et al.*, *J. Plant Bioch. and Biotech.* 6, (2):69-73. 1997.

20 Blackman *et al.*, *Plant Physiol.*, 100:225-230, 1992.

Bol *et al.*, *Annu. Rev. Phytopath.*, 28:113-138, 1990.

Bottjer *et al.*, *Experimental Parasitology*, 60:239-244, 1985.

Bouchez *et al.*, *EMBO Journal*, 8(13):4197-4204, 1989.

Bower *et al.*, *The Plant Journal*, 2:409-416. 1992.

25 Bowler *et al.*, *Ann Rev. Plant Physiol.*, 43:83-116, 1992.

Branson and Guss, *Proceedings North Central Branch Entomological Society of America*, 27:91-95, 1972.

Brears *et al.*, *Plant J.*, 1(2):235-244, 1991.

Brookaert *et al.*, *Science*, 245:1100-1102, 1989.

30 Buchanan-Wollaston *et al.*, *Plant Cell Reports* 11:627-631. 1992

25094423.1



- Buising and Benbow, *Mol Gen Genet*, 243(1):71-81, 1994.
- Callis *et al.*, *Genes Dev.*, 1:1183-1200, 1987.
- Campbell (ed.), *In: Avermectin and Abamectin*, 1989.
- Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 74(2):425-433, 1977.
- 5 Carpenter *et al.*, *Plant Cell*, 4(5):557-571, 1992.
- Casa *et al.*, *Proc. Nat'l Acad. Sci. USA*, 90(23):11212-11216, 1993.
- Cashmore *et al.*, *Gen. Eng. of Plants*, Plenum Press, New York, 29-38, 1983.
- Cech *et al.*, *Cell*, 27:487-496, 1981.
- Chandler *et al.*, *The Plant Cell*, 1:1175-1183, 1989.
- 10 Chau *et al.*, *Science*, 244:174-181, 1989.
- Chen and Wu, *Gene*, 185:195-199, 1997.
- Chilton *et al.*, *Proc. Natl. Acad. Sci. USA*, 71:3672-3676, 1974.
- Chomet *et al.*, *EMBO J.*, 6:295-302, 1987.
- Chowrira *et al.*, *J Biol. Chem.*, 268:19458-62, 1993.
- 15 Chowrira *et al.*, *J. Biol. Chem.*, 269:25856-25864, 1994.
- Christou *et al.*, *Proc. Nat'l Acad. Sci. U S A*, 84(12):3962-3966, 1987.
- Chu *et al.*, *Scientia Sinica*, 18:659-668, 1975..
- Coe *et al.*, *In: Corn and Corn Improvement*, 81-258, 1988.
- Comai *et al.*, *Nature*, 317:741-744, 1985.
- 20 Conkling *et al.*, *Plant Physiol.*, 93:1203-1211, 1990.
- Cordero *et al.*, *Plant J.*, 6(2):141-150, 1994.
- Coruzzi, *Plant Science*, 74:145-155, 1991.
- Coxson *et al.*, *Biotropica*, 24:121-133, 1992.
- Cuozzo *et al.*, *Bio/Technology*, 6:549-553, 1988.
- 25 Cutler *et al.*, *J. Plant Physiol.*, 135:351-354, 1989.
- Czapla and Lang, *J. Econ. Entomol.*, 83:2480-2485, 1990.
- Davies *et al.*, *Plant Physiol.*, 93:588-595, 1990.
- De Block *et al.*, *The EMBO Journal*, 6(9):2513-2518, 1987.
- De Block *et al.*, *Plant Physiol.*, 91:694-701, 1989.

Dellaporta *et al.*, In: *Chromosome Structure and Function: Impact of New Concepts, 18th Stadler Genetics Symposium*, 11:263-282, 1988.

Dennis *et al.*, *Nucl. Acids Res.*, 12(9):3983-4000, 1984.

Depicker *et al.*, *Plant Cell Reports*, 7:63-66, 1988.

5 Dhaese *et al.*, *EMBO Journal*, 2(3):419-426, 1983.

D'Halluin *et al.*, *Plant Cell*, 4(12):1495-1505, 1992.

Ditta *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:7374-7351, 1980.

Dubois *et al.*, *Plant Mol. Biol.*, 31:803-817, 1996.

Dure *et al.*, *Plant Mol. Biol.*, 12:475-486, 1989.

10 Ebert *et al.*, *Proc. Nat'l Acad. Sci. USA*, 84:5745-5749, 1987.

Edwards *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:3459-3463, 1990.

Ehrenshaft *et al.*, *Current Genetics*, 34(6):478-485, 1999.

Eisenberg *et al.*, *Biochimica et Biophysica Acta*, 1477:122-145, 2000.

Ellerstrom *et al.*, *Plant Mol. Biol.*, 32(6):1019-1027, 1996.

15 Ellis *et al.*, *EMBO Journal*, 6(11):3203-3208, 1987.

Enomoto, *et al.*, *J. Bacteriol.*, 6(2):663-668, 1983.

Erdmann *et al.*, *Mol. Jour. Gen. Micro.*, 138:363-368, 1992.

Feinberg and Vogelstein, *Anal. Biochem.*, 132:6-13, 1983.

Finkle *et al.*, *Plant Sci.*, 42:133-140, 1985.

20 Fitzpatrick, *Gen. Engineering News*, 22:7, 1993.

Forde *et al.*, *Plant Cell*, 1:391-401, 1989.

Forde *et al.*, *Plant Cell*, 2:925-939, 1990.

Forster and Symons, *Cell*, 49:211-220, 1987.

Fraley *et al.*, *Bio/Technology*, 3:629-635.

25 Franken *et al.*, *EMBO J.*, 10(9):2605-2612, 1991.

Fransz *et al.*, *Plant Cell Reports*, 8:67-70, 1989.

Fromm *et al.*, *Nature* 319:791-793, 1986

Gal *et al.*, *EMBO J.*, 10:1571-1578, 1991.

Gallusci *et al.*, *Mol. Gen. Genet.*, 244(4):391-400, 1994.

30 Gatehouse *et al.*, *J. Sci. Food. Agric.*, 35:373-380, 1984.

25094423.1

- Gelvin *et al.*, In: *Plant Molecular Biology Manual*, 1990.
- Gerlach *et al.*, *Nature* 328:802-805, 1987.
- Ghosh-Biswas *et al.*, *J. Biotechnol.*, 32(1):1-10, 1994.
- Golic and Lindquist, *Cell*, 59:3, 499-509, 1989.
- 5 Goring *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:1770-1774, 1991.
- Graham *et al.*, *Mol. Cell. Biol.*, 2:1044-1051, 1986.
- Guerrero *et al.*, *Plant Mol. Biol.*, 15:11-26, 1990.
- Gupta *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:1629-1633, 1993.
- Hagio *et al.*, *Plant Cell Rep.*, 10(5):260-264, 1991.
- 10 Hamilton *et al.* *Plant Mol. Biol.*, 18(2):211-218, 1992.
- Hamilton *et al.*, *Proc. Natl. Acad. Sci. USA*, 93(18):9975-9979, 1996.
- Hammock *et al.*, *Nature*, 344:458-461, 1990.
- Haseloff and Gerlach, *Nature*, 334:585-591, 1988.
- Haseloff *et al.*, *Proc. Natl. Acad. Sci. USA*, 94(6):2122-2127, 1997.
- 15 He *et al.*, *Plant Cell Reports*, 14 (2-3):192-196, 1994.
- Hemenway *et al.*, *The EMBO J.*, 7:1273-1280, 1988.
- Hensgens *et al.*, *Plant Mol. Biol.*, 22(6):1101-1127, 1993.
- Hiei *et al.*, *Plant. Mol. Biol.*, 35(1-2):205-218, 1997.
- Hilder *et al.*, *Nature*, 330:160-163, 1987.
- 20 Hinchee *et al.*, *Bio/technol.*, 6:915-922, 1988.
- Holmberg *et al.*, *Nature Biotechnology*, 15(3):244-247, 1997.
- Hou and Lin, *Plant Physiology*, 111(2 Supp.):166, 1996.
- Hudspeth and Grula, *Plant Mol. Biol.*, 12:579-589, 1989.
- Ikeda *et al.*, *J. Bacteriol.*, 169:5615-5621, 1987.
- 25 Ikuta *et al.*, *Bio/technol.*, 8:241-242, 1990.
- Ishida *et al.*, *Nat. Biotechnol.*, 14(6):745-750, 1996.
- Jelesko *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:10302-10307, 1999.
- Jefferson *et al.*, *Proc. Natl. Acad. Sci. USA*, 83(22):8447-8451, 1986.
- Jefferson, *Plant Mol. Biol. Rep.*, 5:387-405, 1987.
- 30 Jeon *et al.*, *Plant Mol. Biol.*, 39(1):35-44, 1999.

- Johnson *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:9871-9875, 1989.
- Joshi, *Nucleic Acids Res.*, 15:6643-6653, 1987.
- Joyce, *Nature*, 338:217-244, 1989.
- Kaasen *et al.*, *J. Bacteriology*, 174:889-898, 1992.
- 5 Kaeppler *et al.*, *Plant Cell Reports* 9: 415-418, 1990.
- Kaeppler *et al.*, *Theor. Appl. Genet.*, 84(5-6):560-566, 1992.
- Karsten *et al.*, *Botanica Marina*, 35:11-19, 1992.
- Katz *et al.*, *J. Gen. Microbiol.*, 129:2703-2714, 1983.
- Keller *et al.*, *EMBO J.*, 8(5):1309-1314, 1989.
- 10 Kelley and Tolan, *Plant Physiol.*, 82:1076-1080, 1986.
- Kiesselbach and Walker, *Am. J. Botany*, 39:561-569, 1952.
- Kim and Cech, *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.
- Klee *et al.*, *Bio-Technology*, 3(7):637-642, 1985.
- Knittel *et al.*, *Plant Cell Reports*, 14(2-3):81-86, 1994.
- 15 Kohler *et al.*, *Plant Mol. Biol.*, 29(6):1293-1298, 1995.
- Koncz *et al.*, *Mol. Gen. Gen.*, 204:383-396, 1986.
- Koster and Leopold, *Plant Physiol.*, 88:829-832, 1988.
- Kriz *et al.*, *Mol. Gen. Genet.*, 207(1):90-98, 1987.
- Kunkel *et al.*, *Methods Enzymol*, 154:367-382, 1987.
- 20 Kyozuka *et al.*, *Plant Cell*, 6(6):799-810, 1994.
- Lam *et al.*, *Ann. Rev. Plant Physiol. Plant Mol Biol.*, 47:569-593, 1996.
- Langridge and Feix, *Cell*, 34:1015-1022, 1983.
- Langridge *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:3219-3223, 1989.
- Laufs *et al.*, *Proc. Natl. Acad. Sci.*, 87(19):7752-7756, 1990.
- 25 Lawton *et al.*, *Plant Mol. Biol.* 9:315-324, 1987.
- Lazzeri, *Methods Mol. Biol.*, 49:95-106, 1995.
- Lee and Saier, *J. of Bacteriol.*, 153(2):685-692, 1983.
- Lee *et al.*, *Korean J. Genet.*, 11(2):65-72, 1989.
- Li *et al.*, *Plant Mol. Biol.*, 23:401-407, 1993.
- 30 Lieber and Strauss, *Mol. Cell. Biol.*, 15: 540-551, 1995.

- Lindstrom *et al.*, *Developmental Genetics*, 11:160-167, 1990.
- Loomis *et al.*, *J. Expt. Zoology*, 252:9-15, 1989.
- Lorz *et al.*, *Mol Gen Genet*, 199:178-182, 1985.
- Lyznik *et al.*, *Maydica*, 27:191-198, 1982.
- 5 Lyznik *et al.*, *Nucleic Acids Res.*, 24(19):3784-3789, 1996.
- Ma *et al.*, *Nature*, 334:631-633, 1988.
- Maeser *et al.*, *Mol. Gen. Genet.*, 230(1-2):170-176, 1991.
- Marcotte *et al.*, *Nature*, 335:454-457, 1988.
- Martinez *et al.*, *J. Mol. Biol.*, 208(4):551-565, 1989.
- 10 McCabe and Martinell, *Bio-Technology*, 11(5):596-598, 1993.
- McCormac *et al.*, *Euphytica*, 1:17-25, 1998.
- McElroy *et al.*, *Plant Cell*, 2:163-171, 1990.
- McGrath and Coruzzi, *Plant Journal*, 1(3):275-280, 1991.
- Michel and Westhof, *J. Mol. Biol.*, 216:585-610, 1990.
- 15 Muhitch, *Physiol. Planta.*, 74:176-180, 1988.
- Muhitch, *Plant Physiol.*, 91:868-875, 1989.
- Muhitch, *Phytochemistry*, 32(5):1125-1130, 1993.
- Muhitch *et al.*, *Plant Physiol.*, 107:757-763, 1995.
- Mundy and Chua, *The EMBO J.*, 7:2279-2286, 1988.
- 20 Murakami *et al.*, *Mol. Gen. Genet.*, 205:42-50, 1986.
- Murashige and Skoog, *Physiol. Plant.*, 15:473-497, 1962.
- Murdock *et al.*, *Phytochemistry*, 29:85-89, 1990.
- Nagatani *et al.*, *Biotech. Tech.*, 11(7):471-473, 1997.
- Napoli *et al.*, *Plant Cell*, 2:279-289, 1990.
- 25 Odell *et al.*, *Nature*, 313:810-812, 1985.
- Ogawa *et al.*, *Sci. Rep.*, 13:42-48, 1973.
- Oliveira *et al.*, *Brazilian J. Med. And Biol. Res.*, 34:567-575, 2001.
- Omirulleh *et al.*, *Plant Mol. Biol.*, 21(3):415-428, 1993.
- Ow *et al.*, *Science*, 234:856-859, 1986.
- 30 Palukaitis *et al.*, *Virology*, 99:145-151, 1979.

Paszkowski *et al.*, *EMBO J.*, 3:2717-2722, 1984.  
 Pearce *et al.*, *Science*, 253:895-898, 1991.  
 Perlak *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:3324-3328, 1991.  
 Perriman *et al.*, *Gene*, 113:157-163, 1992.

5 Peterhans *et al.*, *EMBO J.*, 9(11):3437-3445, 1990.

Phi-Van *et al.*, *Mol. Cell. Biol.*, 10:2302-2307, 1990.

Piatkowski *et al.*, *Plant Physiol.*, 94:1682-1688, 1990.

Porter *et al.*, *Plant Physiol.*, 85:558-565, 1987.

Potrykus *et al.*, *Mol. Gen. Genet.*, 199:183-188, 1985.

10 Poulsen *et al.*, *Mol. Gen. Genet.*, 205(2):193-200, 1986.

Prasher *et al.*, *Biochem. Biophys. Res. Commun.*, 126(3):1259-1268, 1985.

Prody *et al.*, *Science*, 231:1577-1580, 1986.

Quigley *et al.*, *J. Mol. Evol.*, 29(5):412-421, 1989.

Ralston *et al.*, *Genet.*, 119(1):185-197, 1988.

15 Rastogi *et al.*, *Plant Cell. Physiol.*, 39(4):443-446, 1998.

Reed *et al.*, *J. Gen. Microbiology*, 130:1-4, 1984.

Reichel *et al.*, *Proc. Natl. Acad. Sci. USA*, 93(12):5888-5893, 1996.

Reina *et al.*, *Nucl. Acids Res.*, 18(21):6426, 1990.

Reinhold-Hurek and Shub, *Nature*, 357:173-176, 1992.

20 Rensburg *et al.*, *J. Plant Physiol.*, 141:188-194, 1993.

Rhodes *et al.*, *Methods Mol. Biol.*, 55:121-131, 1995.

Ritala *et al.*, *Plant Mol. Biol.*, 24(2):317-325, 1994.

Roberts *et al.*, *Plant J.*, 3(1):111-120, 1993

Rochester, Winer, Shah, *EMBO J.*, 5:451-458, 1986.

25 Rogers *et al.*, *Methods Enzymol.*, 153:253-277, 1987.

Rogers *et al.*, *Plant Molecular Biology Manual A2*:1-12, 1988.

Sakakibara *et al.*, *Plant Cell Physiol.*, 33(1):49-58, 1992.

Sakakibara *et al.*, *J. Biol. Chem.*, 271(47):29561-29568, 1995.

Sakamoto *et al.*, *Plant Mol. Biol.*, 13:611-614, 1989.

Sambrook and Russell in *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2001.

Sarkar *et al.*, *PCR Methods Appl.*, 2:318-322, 1993.

Sauer, *Mol. and Cell. Biol.*, 7: 2087-2096, 1987.

5 Schwob *et al.*, *Plant J.*, 4(3):423-432, 1993.

Senior and Heun, *Genome*, 36(5):884-889.

Shagan and Bar-Zvi, *Plant Physiol.*, 101:1397-1398, 1993.

Shapiro, In: *Mobile Genetic Elements*, 1983.

Sheen *et al.*, *Plant Journal*, 8(5):777-784, 1995.

10 Shure *et al.*, *Cell*, 35:225-233, 1983.

Simpson, *Science*, 233:34-38, 1986.

Singsit *et al.*, *Transgenic Res.*, 6(2):169-176, 1997.

Smith *et al.*, *Mol. Gen. Genet.*, 224:447-481, 1990.

Southern, *J. Mol. Biol.*, 98:503-517, 1975.

15 Spencer *et al.*, *Plant Molecular Biology*, 18:201-210, 1992.

Sprague and Dudley, eds., *Corn and Improvement*, 3rd ed., 1988.

Stalker *et al.*, *Science*, 242:419-422, 1988.

Stief *et al.*, *Nature* 341:343-345, 1989.

Stiefel *et al.*, *Plant Cell*, 2(8):785-793, 1990.

20 Stitt, *Curr. Op. Plant Biol.*, 2:178-186, 1999.

Stromvik *et al.*, *Plant Mol. Biol.*, 41(2):217-231, 1999.

Sullivan, *et al.*, *Mol. Gen. Genet.*, 215(3):431-440, 1989.

Sutcliffe, *Proc. Natl. Acad. Sci. USA*, 75:3737-3741, 1978.

Swoboda *et al.*, *Mol. Gen. Gen.*, 237:33-40, 1993.

25 Swoboda *et al.*, *EMBO J.*, 13:484-489, 1994.

Symons, *Nucl. Acids Res.*, 9(23):6527-6537, 1981.

Symons, *Annu. Rev. Biochem.*, 61:641-671, 1992.

Szostak *et al.*, *Cell*, 33:25-35, 1983.

Tanksley *et al.*, *Bio/Technology*, 7:257-264, 1989.

30 Taramino and Tingey, *Genome*, 39(2):277-287, 1996.

- Tarczynski *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:1-5, 1992.
- Tarczynski *et al.*, *Science*, 259:508-510, 1993.
- Tenaillon *et al.*, *Proc. Natl. Acad. Sci. USA*, 98(16):9161-9166, 2001.
- Thillet *et al.*, *J. Biol. Chem.*, 263:12500-12508, 1988.
- 5 Thompson *et al.*, *The EMBO Journal*, 6(9):2519-2523, 1987.
- Thompson *et al.*, *Euphytica*, 85(1-3):75-80, 1995.
- Thorne, *Ann. Rev. Plant Physiol.*, 36:317-343, 1985.
- Tian *et al.*, *Plant Cell Rep.*, 16:267-271, 1997.
- Timmermans *et al.*, *J. Biotechnol.*, 14:333-344, 1990.
- 10 Tingay *et al.*, *The Plant Journal*, 11(6):1369-1376, 1997.
- Tingey *et al.*, *EMBO J.*, 6(1):1-9, 1987.
- Tingey *et al.*, *J. Biol. Chem.*, 263(20):9651-9657, 1988.
- Tinland *et al.*, *EMBO J.*, 14(14):3585-3595, 1995.
- Tobin and Yamaya, *J. Exp. Botany*, 52(356):591-604, 2001.
- 15 Tomes *et al.*, *Plant. Mol. Biol.* 14(2):261-268, 1990.
- Tomic *et al.*, *Nucl. Acids Res.*, 18:1656, 1990.
- Torbet *et al.*, *Crop Science*, 38(1):226-231, 1998.
- Torbet *et al.*, *Plant Cell Reports*, 14(10):635-640, 1995.
- Toriyama *et al.*, *Theor Appl. Genet.*, 73:16, 1986.
- 20 Tsukada *et al.*, *Plant Cell Physiol.*, 30(4):599-604, 1989.
- Twell *et al.*, *Plant Physiol.*, 91:1270-1274, 1989.
- Twell *et al.*, *Development*, 109(3):705-713, 1990.
- Tyagi and Kramer, *Nature Biotech.*, 14:303-308, 1996.
- Uchimiya *et al.*, *Mol. Gen. Genet.*, 204:204-207, 1986.
- 25 Ugaki *et al.*, *Nucl. Acid Res.*, 19:371-377, 1991.
- Upender *et al.*, *Biotechniques* 18(1):29-30, 1995.
- Uribe *et al.*, *Plant Mol. Biol.*, 37(6):1069-1078, 1998.
- Van der Krol *et al.*, *Plant Cell*, 2:291-299, 1990.
- Van der Meer *et al.*, *Plant Mol. Biol.*, 15(1):95-109, 1990.
- 30 Van Eck *et al.*, *Plant Cell Reports*, 14(5):299-304, 1995.



- Van Tunen *et al.*, *EMBO J.*, 7(5):1257-1263, 1988.
- Vasil *et al.*, *Plant Physiol.*, 91:1575-1579, 1989.
- Vernon and Bohnert, *The EMBO J.*, 11:2077-2085, 1992.
- Vodkin *et al.*, *Cell*, 34:1023-31, 1983.
- 5 Vogel *et al.*, *J. Cell. Biochem.*, (Suppl. 0) 13:Part D, 1989.
- Walker *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:6624-6628, 1987.
- Wandelt and Feix, *Nucl. Acids Res.*, 17(6):2354, 1989.
- Wang *et al.*, *Molecular and Cellular Biology*, 12(8):3399-3406, 1992.
- Watrud *et al.*, In: *Engineered Organisms and the Environment*, 1985.
- 10 Watson and Ramstad, eds., *Corn: Chemistry and Technology*, 1987.
- Weber *et al.*, *BioTechniques*, 25(3):415-419, 1988.
- Wen-jun and Forde, *Nucl. Acids. Res.* 17:8385, 1989.
- Wenzler *et al.*, *Plant Mol. Biol.*, 12:41-50, 1989.
- Withers and King, *Plant Physiol.*, 64:675-678, 1979.
- 15 Wolter *et al.*, *The EMBO J.*, 11(13):4685-4692, 1992.
- Xiang and Guerra, *Plant Physiol.*, 102:287-293, 1993.
- Xu *et al.*, *Plant Physiol.*, 110:249-257, 1996.
- Yamada *et al.*, *Plant Cell Rep.*, 4:85-88, 1986.
- Yamaguchi-Shinozaki *et al.*, *Plant Cell Physiol.*, 33:217-224, 1992.
- 20 Yang and Russell, *Proc. Natl. Acad. Sci. USA*, 87:4144-4148, 1990.
- Yuan and Altman, *Science*, 263:1269-1273, 1994.
- Yuan *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:8006-8010, 1992.
- Zhang *et al.*, *Mol. Biotechnology*, 8:223-231, 1997.
- Zheng and Edwards, *J. Gen. Virol.*, 71:1865-1868, 1990.
- 25 Zhou *et al.*, *Plant Cell Reports*, 12(11):612-616, 1993.
- Zubko *et al.*, *Nature Biotech.*, 18:442-445, 2000.
- Zukowsky *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:1101-1105, 1983.

\* \* \*



All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000  
1001  
1002  
1003  
1004  
1005  
1006  
1007  
1008  
1009  
1010  
1011  
1012  
1013  
1014  
1015  
1016  
1017  
1018  
1019  
1020  
1021  
1022  
1023  
1024  
1025  
1026  
1027  
1028  
1029  
1030  
1031  
1032  
1033  
1034  
1035  
1036  
1037  
1038  
1039  
1040  
1041  
1042  
1043  
1044  
1045  
1046  
1047  
1048  
1049  
1050  
1051  
1052  
1053  
1054  
1055  
1056  
1057  
1058  
1059  
1060  
1061  
1062  
1063  
1064  
1065  
1066  
1067  
1068  
1069  
1070  
1071  
1072  
1073  
1074  
1075  
1076  
1077  
1078  
1079  
1080  
1081  
1082  
1083  
1084  
1085  
1086  
1087  
1088  
1089  
1090  
1091  
1092  
1093  
1094  
1095  
1096  
1097  
1098  
1099  
1100  
1101  
1102  
1103  
1104  
1105  
1106  
1107  
1108  
1109  
1110  
1111  
1112  
1113  
1114  
1115  
1116  
1117  
1118  
1119  
1120  
1121  
1122  
1123  
1124  
1125  
1126  
1127  
1128  
1129  
1130  
1131  
1132  
1133  
1134  
1135  
1136  
1137  
1138  
1139  
1140  
1141  
1142  
1143  
1144  
1145  
1146  
1147  
1148  
1149  
1150  
1151  
1152  
1153  
1154  
1155  
1156  
1157  
1158  
1159  
1160  
1161  
1162  
1163  
1164  
1165  
1166  
1167  
1168  
1169  
1170  
1171  
1172  
1173  
1174  
1175  
1176  
1177  
1178  
1179  
1180  
1181  
1182  
1183  
1184  
1185  
1186  
1187  
1188  
1189  
1190  
1191  
1192  
1193  
1194  
1195  
1196  
1197  
1198  
1199  
1200  
1201  
1202  
1203  
1204  
1205  
1206  
1207  
1208  
1209  
1210  
1211  
1212  
1213  
1214  
1215  
1216  
1217  
1218  
1219  
1220  
1221  
1222  
1223  
1224  
1225  
1226  
1227  
1228  
1229  
1230  
1231  
1232  
1233  
1234  
1235  
1236  
1237  
1238  
1239  
1240  
1241  
1242  
1243  
1244  
1245  
1246  
1247  
1248  
1249  
1250  
1251  
1252  
1253  
1254  
1255  
1256  
1257  
1258  
1259  
1260  
1261  
1262  
1263  
1264  
1265  
1266  
1267  
1268  
1269  
1270  
1271  
1272  
1273  
1274  
1275  
1276  
1277  
1278  
1279  
1280  
1281  
1282  
1283  
1284  
1285  
1286  
1287  
1288  
1289  
1290  
1291  
1292  
1293  
1294  
1295  
1296  
1297  
1298  
1299  
1300  
1301  
1302  
1303  
1304  
1305  
1306  
1307  
1308  
1309  
1310  
1311  
1312  
1313  
1314  
1315  
1316  
1317  
1318  
1319  
1320  
1321  
1322  
1323  
1324  
1325  
1326  
1327  
1328  
1329  
1330  
1331  
1332  
1333  
1334  
1335  
1336  
1337  
1338  
1339  
1340  
1341  
1342  
1343  
1344  
1345  
1346  
1347  
1348  
1349  
1350  
1351  
1352  
1353  
1354  
1355  
1356  
1357  
1358  
1359  
1360  
1361  
1362  
1363  
1364  
1365  
1366  
1367  
1368  
1369  
1370  
1371  
1372  
1373  
1374  
1375  
1376  
1377  
1378  
1379  
1380  
1381  
1382  
1383  
1384  
1385  
1386  
1387  
1388  
1389  
1390  
1391  
1392  
1393  
1394  
1395  
1396  
1397  
1398  
1399  
1400  
1401  
1402  
1403  
1404  
1405  
1406  
1407  
1408  
1409  
1410  
1411  
1412  
1413  
1414  
1415  
1416  
1417  
1418  
1419  
1420  
1421  
1422  
1423  
1424  
1425  
1426  
1427  
1428  
1429  
1430  
1431  
1432  
1433  
1434  
1435  
1436  
1437  
1438  
1439  
1440  
1441  
1442  
1443  
1444  
1445  
1446  
1447  
1448  
1449  
1450  
1451  
1452  
1453  
1454  
1455  
1456  
1457  
1458  
1459  
1460  
1461  
1462  
1463  
1464  
1465  
1466  
1467  
1468  
1469  
1470  
1471  
1472  
1473  
1474  
1475  
1476  
1477  
1478  
1479  
1480  
1481  
1482  
1483  
1484  
1485  
1486  
1487  
1488  
1489  
1490  
1491  
1492  
1493  
1494  
1495  
1496  
1497  
1498  
1499  
1500  
1501  
1502  
1503  
1504  
1505  
1506  
1507  
1508  
1509  
1510  
1511  
1512  
1513  
1514  
1515  
1516  
1517  
1518  
1519  
1520  
1521  
1522  
1523  
1524  
1525  
1526  
1527  
1528  
1529  
1530  
1531  
1532  
1533  
1534  
1535  
1536  
1537  
1538  
1539  
1540  
1541  
1542  
1543  
1544  
1545  
1546  
1547  
1548  
1549  
1550  
1551  
1552  
1553  
1554  
1555  
1556  
1557  
1558  
1559  
1560  
1561  
1562  
1563  
1564  
1565  
1566  
1567  
1568  
1569  
1570  
1571  
1572  
1573  
1574  
1575  
1576  
1577  
1578  
1579  
1580  
1581  
1582  
1583  
1584  
1585  
1586  
1587  
1588  
1589  
1590  
1591  
1592  
1593  
1594  
1595  
1596  
1597  
1598  
1599  
1600  
1601  
1602  
1603  
1604  
1605  
1606  
1607  
1608  
1609  
1610  
1611  
1612  
1613  
1614  
1615  
1616  
1617  
1618  
1619  
1620  
1621  
1622  
1623  
1624  
1625  
1626  
1627  
1628  
1629  
1630  
1631  
1632  
1633  
1634  
1635  
1636  
1637  
1638  
1639  
1640  
1641  
1642  
1643  
1644  
1645  
1646  
1647  
1648  
1649  
1650  
1651  
1652  
1653  
1654  
1655  
1656  
1657  
1658  
1659  
1660  
1661  
1662  
1663  
1664  
1665  
1666  
1667  
1668  
1669  
1670  
1671  
1672  
1673  
1674  
1675  
1676  
1677  
1678  
1679  
1680  
1681  
1682  
1683  
1684  
1685  
1686  
1687  
1688  
1689  
1690  
1691  
1692  
1693  
1694  
1695  
1696  
1697  
1698  
1699  
1700  
1701  
1702  
1703  
1704  
1705  
1706  
1707  
1708  
1709  
1710  
1711  
1712  
1713  
1714  
1715  
1716  
1717  
1718  
1719  
1720  
1721  
1722  
1723  
1724  
1725  
1726  
1727  
1728  
1729  
1730  
1731  
1732  
1733  
1734  
1735  
1736  
1737  
1738  
1739  
1740  
1741  
1742  
1743  
1744  
1745  
1746  
1747  
1748  
1749  
1750  
1751  
1752  
1753  
1754  
1755  
1756  
1757  
1758  
1759  
1760  
1761  
1762  
1763  
1764  
1765  
1766  
1767  
1768  
1769  
1770  
1771  
1772  
1773  
1774  
1775  
1776  
1777  
1778  
1779  
1780  
1781  
1782  
1783  
1784  
1785  
1786  
1787  
1788  
1789  
1790  
1791  
1792  
1793  
1794  
1795  
1796  
1797  
1798  
1799  
1800  
1801  
1802  
1803  
1804  
1805  
1806  
1807  
1808  
1809  
1810  
1811  
1812  
1813  
1814  
1815  
1816  
1817  
1818  
1819  
1820  
1821  
1822  
1823  
1824  
1825  
1826  
1827  
1828  
1829  
1830  
1831  
1832  
1833  
1834  
1835  
1836  
1837  
1838  
1839  
1840  
1841  
1842  
1843  
1844  
1845  
1846  
1847  
1848  
1849  
1850  
1851  
1852  
1853  
1854  
1855  
1856  
1857  
1858  
1859  
1860  
1861  
1862  
1863  
1864  
1865  
1866  
1867  
1868  
1869  
1870  
1871  
1872  
1873  
1874  
1875  
1876  
1877  
1878  
1879  
1880  
1881  
1882  
1883  
1884  
1885  
1886  
1887  
1888  
1889  
1890  
1891  
1892  
1893  
1894  
1895  
1896  
1897  
1898  
1899  
1900  
1901  
1902  
1903  
1904  
1905  
1906  
1907  
1908  
1909  
1910  
1911  
1912  
1913  
1914  
1915  
1916  
1917  
1918  
1919  
1920  
1921  
1922  
1923  
1924  
1925  
1926  
1927  
1928  
1929  
1930  
1931  
1932  
1933  
1934  
1935  
1936  
1937  
1938  
1939  
1940  
1941  
1942  
1943  
1944  
1945  
1946  
1947  
1948  
1949  
1950  
1951  
1952  
1953  
1954  
1955  
1956  
1957  
1958  
1959  
1960  
1961  
1962  
1963  
1964  
1965  
1966  
1967  
1968  
1969  
1970  
1971  
1972  
1973  
1974  
1975  
1976  
1977  
1978  
1979  
1980  
1981  
1982  
1983  
1984  
1985  
1986  
1987  
1988  
1989  
1990  
1991  
1992  
1993  
1994  
1995  
1996  
1997  
1998  
1999  
2000  
2001  
2002  
2003  
2004  
2005  
2006  
2007  
2008  
2009  
2010  
2011  
2012  
2013  
2014  
2015  
2016  
2017  
2018  
2019  
2020  
2021  
2022  
2023  
2024  
2025  
2026  
2027  
2028  
2029  
2030  
2031  
2032  
2033  
2034  
2035  
2036  
2037  
2038  
2039  
2040  
2041  
2042  
2043  
2044  
2045  
2046  
2047  
2048  
2049  
2050  
2051  
2052  
2053  
2054  
2055  
2056  
2057  
2058  
2059  
2060  
2061  
2062  
2063  
2064  
2065  
2066  
2067  
2068  
2069  
2070  
2071  
2072  
2073  
2074  
2075  
2076  
2077  
2078  
2079  
2080  
2081  
2082  
2083  
2084  
2085  
2086  
2087  
2088  
2089  
2090  
2091  
2092  
2093  
2094  
2095  
2096  
2097  
2098  
2099  
2100  
2101  
2102  
2103  
2104  
2105  
2106  
2107  
2108  
2109  
2110  
2111  
2112  
2113  
2114  
2115  
2116  
2117  
2118  
2119  
2120  
2121  
2122  
2123  
2124  
2125  
2126  
2127  
2128  
2129  
2130  
2131  
2132  
2133  
2134  
2135  
2136  
2137  
2138  
2139  
2140  
2141  
2142  
2143  
2144  
2145  
2146  
2147  
2148  
2149  
2150  
2151  
2152  
2153  
2154  
2155  
2156  
2157  
2158  
2159  
2160  
2161  
2162  
2163  
2164  
2165  
2166  
2167  
2168  
2169  
2170  
2171  
2172  
2173  
2174  
2175  
2176  
2177  
2178  
2179  
2180  
2181  
2182  
2183  
2184  
2185  
2186  
2187  
2188  
2189  
2190  
2191  
2192  
2193  
2194  
2195  
2196  
2197  
2198  
2199  
2200  
2201  
2202  
2203  
2204  
2205  
2206  
2207  
2208  
2209  
2210